Conserv Genet (2015) 16:595–611
DOI 10.1007/s10592-014-0685-x

RESEARCH ARTICLE

Toll-like receptor diversity in 10 threatened bird species: relationship with microsatellite heterozygosity

Catherine E. Grueber · Gabrielle J. Knafler · Tania M. King · Alistair M. Senior · Stefanie Grosser · Bruce Robertson · Kerry A. Weston · Patricia Brekke · Christian L. W. Harris · Ian G. Jamieson

Received: 6 August 2014 / Accepted: 19 November 2014 / Published online: 8 January 2015

Abstract Measuring individual-level heterozygosity in threatened species is one approach to understanding and mitigating losses of genetic diversity and the role of inbreeding depression in those populations. In many conservation contexts, this goal is approached by assaying levels of microsatellite diversity, and inference is often extended to functional genomic regions. Our study quantifies diversity of innate immunity toll-like receptor (TLR) genes in 10 threatened New Zealand birds across four avian orders, with an average of 20.1 individuals and 6.2 TLR loci (sequences averaging 850 bp in length) per species. We provide detailed TLR diversity statistics for these 10 species, which showed more evidence for genetic drift than balancing selection at TLR loci, with two possible exceptions (*TLR1LA* for hihi and *TLR5* for kokako). Our observations also support a possible gene-duplication of *TLR7* in rock wren, indicating that a *TLR7* duplication previously observed in other passerines may have occurred early in the divergence of this order. In addition to these analyses of population-level TLR sequence diversity, we used an average of 14.6 polymorphic microsatellite loci per species to study, for the first time, the relationship between microsatellite internal relatedness (a measure of individual homozygosity) and TLR heterozygosity. There was no relationship between microsatellite and TLR heterozygosity of individuals within species, suggesting that the predictive power of microsatellites to evaluate functional diversity is poor, and highlighting the value of adding data from putatively functional genomic regions, such as TLRs, in the study of genetic diversity of threatened species. Overall this study provides valuable data for comparison with more widespread species, and facilitates research into the importance of TLR diversity in natural populations of conservation concern.

Keywords Apterygiformes · Conservation · Gruiformes · Immunity genes · Inbreeding · Passeriformes · Psittaciformes

Electronic supplementary material The online version of this article (doi:10.1007/s10592-014-0685-x) contains supplementary material, which is available to authorized users.

C. E. Grueber · G. J. Knafler · T. M. King · S. Grosser · B. Robertson · K. A. Weston · I. G. Jamieson
Allan Wilson Centre for Molecular Ecology and Evolution, Department of Zoology, University of Otago, Dunedin, New Zealand

C. E. Grueber (✉)
Faculty of Veterinary Science, University of Sydney, Sydney, NSW 2006, Australia
e-mail: catherine.grueber@sydney.edu.au

A. M. Senior
Charles Perkins Centre and School of Biological Sciences, University of Sydney, Sydney, NSW, Australia

P. Brekke
Institute of Zoology, Zoological Society of London, London, UK

C. L. W. Harris
Department of Biochemistry, University of Otago, Dunedin, New Zealand

Introduction

Studying genetic variation among individuals is a core goal of evolutionary biology and crucial to conservation genetics. Population bottlenecks and subsequent inbreeding can result in loss of genetic diversity both at the population (allelic) and individual (observed heterozygosity) levels.

Loss of heterozygosity can lead to inbreeding depression via losses of heterozygote advantage and increased probability of homozygosity for deleterious recessive alleles (Allendorf and Luikart 2007). Left unmanaged, inbreeding depression has the potential to undermine population restoration goals through negative impacts on both individual and population viability (Keller and Waller 2002). Measuring (and thus mitigating) losses of heterozygosity in wild populations is frequently based on individual-level heterozygosity (multilocus heterozygosity, MLH) evaluated using neutral genetic markers such as microsatellites (Slate et al. 2004). Many studies of threatened species still rely on neutral loci, such as microsatellites, at least for initial estimates of heterozygosity (Kirk and Freeland 2011). Despite their common usage, the suitability of microsatellites to inform patterns at functional loci is debated (Väli et al. 2008; Chapman et al. 2009; Ljungqvist et al. 2010; Szulkin et al. 2010), so a growing number of studies also include diversity at functional loci when evaluating changes in heterozygosity.

Immune genes are ideal for studying functionally relevant genetic diversity as they represent the most rapidly-evolving parts of the genome, due to diverse and constant selection pressure from co-evolving pathogens (Hedrick 1998; Piertney and Oliver 2006; Deakin 2012). Studies of putatively functional genetic diversity usually examine genes of the major histocompatibility complex (MHC), which encode cell-surface proteins that bind and present foreign peptides for the initiation of cell-mediated immunity (Piertney and Oliver 2006; Spurgin and Richardson 2010). Although MHC is well-studied in the molecular ecology literature, there is a growing awareness that non-MHC parts of the immune system can provide useful insight into processes affecting adaptive diversity of non-model organisms (Jepson et al. 1997; Acevedo-Whitehouse and Cunningham 2006; Vinkler and Albrecht 2009; Bollmer et al. 2011). Furthermore, calculating locus-specific MHC genotypes for non-model species, especially birds, can be technically challenging. The MHC is highly duplicated in many species, particularly passerine birds (Westerdahl 2007; Bollmer et al. 2010), often necessitating cloning or next generation sequencing (Babik et al. 2009), the latter with accompanying specialised analyses (e.g. Galan et al. 2010; Meglécz et al. 2011). These technical challenges can limit our ability to determine MHC locus-specific heterozygosity for individual animals.

Recently, a family of innate-immunity genes, the toll-like receptors (TLRs), have been used to address questions of ecological relevance in wild populations (Turner et al. 2011; Grueber et al. 2012, 2013). TLRs are a family of genes encoding proteins that recognise a wide diversity of pathogens through binding of conserved pathogen-associated molecular patterns (Uematsu and Akira 2008). Through intra-cellular signalling, TLRs initiate innate and adaptive aspects of the immune response (Barreiro et al. 2009). TLRs offer several advantages over MHC for assaying immune-gene heterozygosity, one of which is that gene duplications are either rare or well-characterised (Temperley et al. 2008; Grueber et al. 2012). Individual TLR genotypes can therefore be obtained using routine Sanger sequencing of PCR amplicons (current study; Alcaide and Edwards 2011; Grueber et al. 2012).

Previous analyses of avian TLR sequence diversity found evidence that a number of codons in most TLR genes experience pervasive or episodic positive selection (Grueber et al. 2014). Where previous studies have used a phylogenetic approach, with only 1–2 samples per species, the current study focuses on within-species tests of selection, which require population-level data. Here we determine levels of TLR heterozygosity of individuals from 10 threatened New Zealand bird species (across four avian orders; "Appendix" section). We first report detailed TLR diversity statistics within these populations of New Zealand birds, and test for evidence of balancing selection on these key immunity genes. Because microsatellites are still widely used for evaluating patterns of genetic diversity in threatened populations, this study also evaluates whether microsatellite data can predict levels of TLR heterozygosity of individuals within populations. Additionally, should a relationship between microsatellites and other types of heterozygosity exist, it may be population-specific due to variation in demographic processes (Grueber et al. 2008b; Alho et al. 2009), thus our models enable us to examine the consistency of relationships between neutral and functional heterozygosity across species.

Methods

Study species and DNA samples

Background data for the study species included here, as well as details of the sites from which our samples were sourced, are provided in "Appendix" section and Fig. 1. All samples used herein had been collected for previous studies and made available for this study either as extracted DNA or as tissue samples [whole blood in ethanol or lysis buffer (Seutin et al. 1991), or feathers] (see "Appendix" section for the sources of samples used here). DNA was purified from tissue samples using a modified Chelex (Bio-Rad) extraction (Walsh et al. 1991; Casquet et al. 2012). Feather sample extractions were followed by an additional $LiCl_2$ and ethanol precipitation using GenElute linearised polyacrylamide (Sigma) as a DNA carrier. Extracted DNA was stored at 4 °C (short-term, <1 year) or −20 °C (long-term).

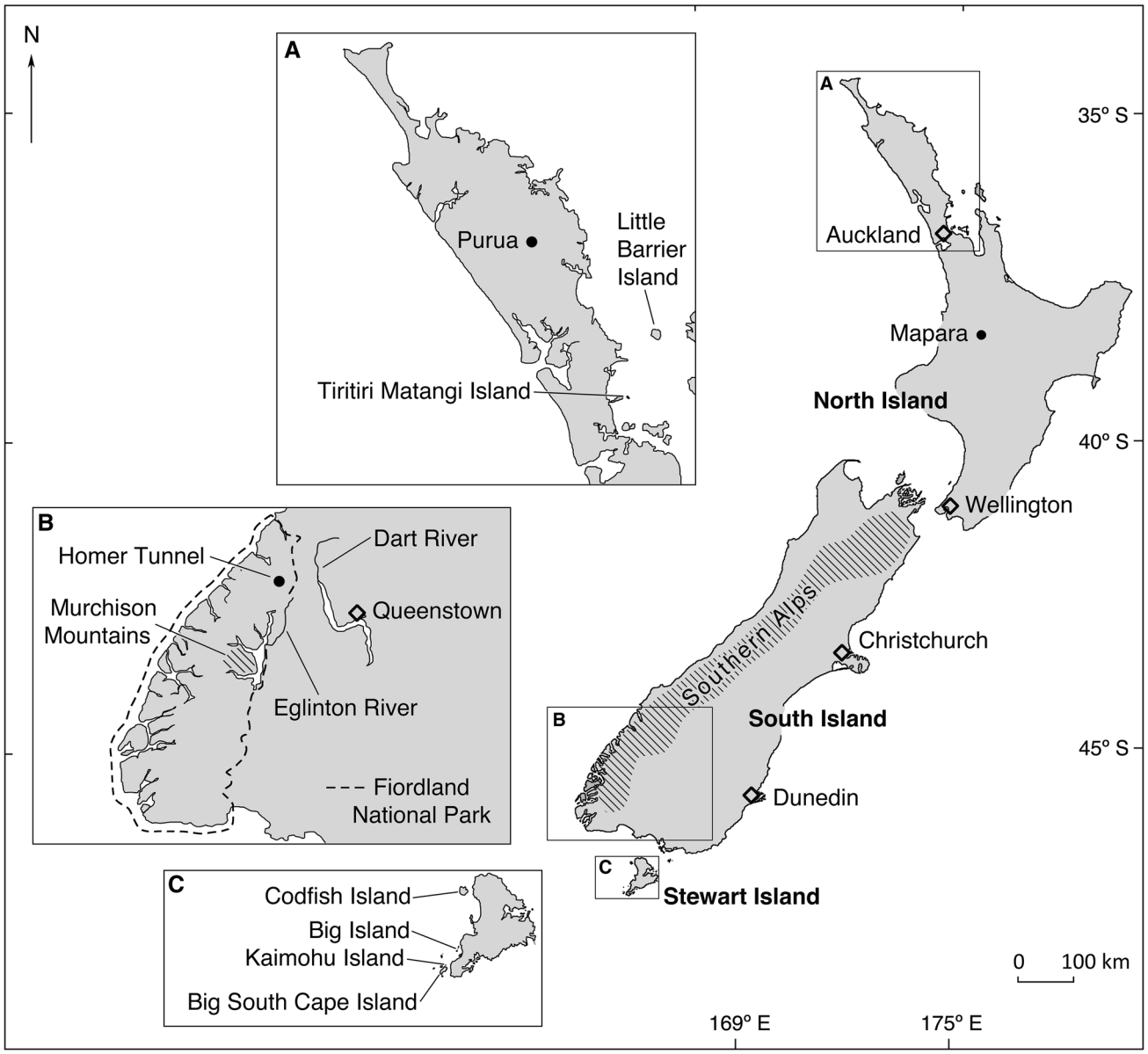

Fig. 1 Important sites for the conservation of the species studied herein ("Appendix" section). Major cities of New Zealand are labelled with *open diamonds*, localities of interest with *filled circles*. *Insets* show the Northland (*A*), Fiordland (*B*) and Stewart Island (*C*) regions; the location of the Southern Alps (*dashed area* on main map) is an approximation

TLR sequencing

In birds, there are 10 known TLR genes, which bind a variety of pathogen-associated molecular patterns (Cormican et al. 2009). We used previously published primers (Alcaide and Edwards 2011; Grueber et al. 2012; Grueber and Jamieson 2013) to amplify a minimum of five TLR loci per species; all loci were amplified in at least five species (see "Results" section). For each TLR gene, we amplified an average of 850 bp targeting the extracellular leucine-rich repeat region (LRR) of each gene (Alcaide and Edwards 2011; Grueber et al. 2014). These regions were targeted because the LRR domain is associated with pathogen recognition (Werling et al. 2009), and is expected to show greatest sensitivity to pathogen-driven selection (Areal et al. 2011; Grueber et al. 2014). Amplification, clean-up and sequencing followed published protocols (Grueber and Jamieson 2013). Sanger sequencing service was provided by Macrogen Inc. (Korea) and the Genetic Analysis Service at the University of Otago (New Zealand). Sequence editing and haplotype reconstruction protocols are provided in Supplementary Methods; all SNPs (synonymous and nonsynonymous) were used in the construction of haplotypes. Haplotypes were assigned 2-digit

codes and used as genotype data for calculating heterozygosity.

TLR diversity and inference of selection

Balancing selection can result in an excess of heterozygotes, so we tested our TLR data for deviation from Hardy–Weinberg equilibrium using the exact test implemented in Arlequin v3.5.1.3 (Excoffier and Lischer 2010), with 1×10^6 steps in the Markov chain following 1×10^5 burn-in iterations. Inferred haplotypes were also used to calculate polymorphism statistics using DNAsp (Librado and Rozas 2009), including number of inferred haplotypes (h), mean nucleotide differences between haplotypes (k), nucleotide diversity (π), and numbers of non-synonymous and synonymous single-nucleotide polymorphisms (SNPs).

We used Arlequin to evaluate summary statistics of selection for those genes that showed at least five haplotypes within a species (following Alcaide and Edwards 2011). The Ewens–Watterson test (Ewens 1972; Watterson 1978) examines whether the frequency of alleles in the sample are more uniform than predicted under neutrality, interpreted as a form of balancing selection (Spurgin and Richardson 2010). Tajima's D (Tajima 1989) can be considered analogous to the Ewens–Watterson test, but explicitly accounts for mutational events and therefore the level of divergence among alleles, rather than simply their frequencies (Garrigan and Hedrick 2003). For both tests, P values were evaluated by 1,000 permutations in Arlequin. Both of these metrics can be influenced by demographic population processes, meaning comparisons between species/populations are problematic (Nei 1987). We therefore restrict our inference to comparisons among loci within species (as they should be similarly affected by demographic processes); four species had $\geq$2 loci with $h \geq 5$: kakariki, rock wren, robin and kokako.

TLRs that bind different types of pathogen-derived ligands may evolve in different ways; we thus compared levels of diversity (SNPs, h, π and k) between TLRs proposed to bind viral ligands (*TLR3*, *TLR7*, *TLR21*) and those proposed to bind proteinaceous ligands (*TLR1LA*, *TLR1LB*, *TLR2B*, *TLR4*, *TLR5*, *TLR15*) (Areal et al. 2011; Keestra et al. 2013). These comparisons were performed using generalised linear mixed modelling with lme4 v1.0-5 (Bates and Maechler 2009) in R v3.0.1 (R Core Team 2013). Our fixed factor was a binary predictor of "viral"/"non-viral" (1/0) and we included diversity data from each TLR locus as the response variable, fitting "species" as a random factor to account for mean differences in diversity between species. Each model used an error structure appropriate for the response variable: count data (h and number of SNPs) were fitted with a Poisson error distribution; data derived from count-based measures (k) were log transformed and fitted with a Gaussian error distribution; proportion-based data (π) were logit transformed and fitted with a Gaussian error distribution. For number of SNPs, we examined total SNPs, as well as non-synonymous and synonymous SNPs separately. Fitted values were obtained from our models using functions available in the R package arm v1.6-10 (Gelman et al. 2009); inference was based on the effect size of the slope and its associated 95 % confidence interval (1.96 $\times$ SE of the slope).

Microsatellite data

Microsatellite data for the individuals included here were sourced from published studies, our own on-going research, and we specifically obtained microsatellite genotypes from kiwi and kokako. Full microsatellite genotyping protocols for kiwi and kokako are detailed in Supplementary Methods. Data from 8 to 25 microsatellite loci per species were used; heterozygosity summary statistics and references for all microsatellite data are provided in "Results" section. We note that different microsatellite loci were typed in each species; our microsatellite datasets are therefore not directly comparable between species, but can be used to evaluate relationships *within* species (see below).

Individual MLH was calculated from the microsatellite data using the R-package Rhh (Alho et al. 2010); these calculations were performed by analysing the microsatellite dataset for each species separately. We used the MLH metric internal relatedness (IR), as this measure is intended as a DNA-based measure of an individual's inbreeding coefficient (Amos et al. 2001). Note that IR is a measure of homozygosity and therefore is expected to be negatively correlated with other measures of heterozygosity. For comparison, we also evaluated an alternative microsatellite MLH metric, standardised heterozygosity (SH) (Coltman et al. 1999). IR and SH were highly correlated in our dataset ($r = -0.929$; $N = 216$ individuals), and our main results from both were qualitatively similar. We therefore present only the results using IR, as this measure is commonly used in studies that measure MLH with a view to informing patterns of inbreeding (i.e. studies operating in a conservation context similar to ours).

Relationship between microsatellite and TLR heterozygosities

We examined the ability of microsatellite MLH to predict TLR heterozygosity of individuals of each species using a GLMM implemented by the *MCMCglmm* function in the R-package MCMCglmm (Hadfield 2010). In the GLMM, the response variable was the proportion of TLR loci

genotyped as heterozygous for each individual, specified in the model as the per-individual counts of heterozygous and homozygous TLR genotypes. Given that our response variable is a proportion, the model was estimated with a logit link function (specified in MCMCglmm as *family* = "*Multinomial2*"). Microsatellite IR was our fixed predictor variable. To assess whether the ability of microsatellite IR to predict TLR heterozygosity was species-specific, we also fitted a random effect with 10 levels, specifying the species of each individual, with a random slope for microsatellite IR. This model allows us to estimate two processes: (1) the between-species variance associated with the slope of TLR heterozygosity on microsatellite IR, and its associated error; (2) species-specific slopes of TLR heterozygosity on microsatellite IR, and associated errors, estimated with partial pooling. The estimation of error on these values is a specific advantage of using the MCMCglmm package. Detailed MCMC specifications, convergence diagnostics and sensitivity analyses are provided in Supplementary Methods.

Results

TLR diversity in 10 bottlenecked New Zealand species

Across all study species and genes, we obtained population-level data (total 1,225 sequences) for a total of 18,168 codons of TLR sequence (Table 1). The length of the sequenced region for each gene/species combination varied, as a range of PCR primers were used, although sequences from the same gene showed a high degree of overlap between species (compare starting positions referenced against the chicken genome, Table 1). We sequenced a mean of 20.1 individuals per species per gene (range 17–24 individuals), a mean of 6.2 genes per species (range 5 [kiwi, kakapo, hihi, rock wren] to 8 [robin, saddleback] genes) and each gene was sequenced in a mean of 6.9 species (range 5 [*TLR7*, *TLR15*] to 10 [*TLR1LA*] species) (Table 1).

All sequenced TLR regions were polymorphic in kiwi ($N = 5$ loci), kakariki ($N = 5$ loci) and robin ($N = 8$ loci); all species were polymorphic at one or more TLR loci (Table 1). *TLR1LA* showed the highest rate of polymorphism, being variable in 9 of 10 (90 %) species sequenced, followed by *TLR4* and *TLR15* (both variable in 5 of 6 [83 %] species sequenced) (Table 1). *TLR21* and *TLR2B* showed the lowest rates of polymorphism (for both loci, 40 % of sequenced species were variable) (Table 1). We detected 219 SNPs in total over all loci and species; there were slightly more non-synonymous sequence variants (112) than synonymous variants (109) (Table 1). We observed a mean of 5.34 SNPs per polymorphic alignment, although there was considerable variation in this statistic (SD = 4.89; $N = 41$ species–gene alignments).

None of our alignments showed frame-shift mutation, indicating no evidence of potential pseudogenisation that has been previously observed in some passerine *TLR5* alignments (Alcaide and Edwards 2011; Bainová et al. 2014). Only one alignment (hihi *TLR21*) appeared to show indel variation within species; the chromatogram data for two individuals appeared to show a heterozygote 3-bp indel polymorphism in two independent PCRs each. Cloning or next-generation sequencing data would be required to confirm this indel variant; *TLR21* data for these two individual hihi were excluded from subsequent analyses. *TLR7* has previously been observed to be duplicated in passerine birds (Cormican et al. 2009; Alcaide and Edwards 2011; Grueber et al. 2012; Hartmann et al. 2014); herein *TLR7* sequence data were obtained for two passerines: saddleback and rock wren. The *TLR7* sequencing chromatograms for all saddleback samples ($N = 20$) appeared to show heterozygosity at five nucleotide sites. This complete heterozygosity is suggestive of a duplication of *TLR7*, if the five "variable" sites comprise differences between the two copies of the gene, rather than heterozygosity *per se*; cloning would be required to confirm this hypothesis.

New Zealand rock wren is of particular interest for *TLR7*, as the species belongs to the ancient family Acanthisittidae, which is phylogenetically distinct from all other passerines (Ericson et al. 2002). It is therefore informative to determine whether rock wren, as a representative of this group, show a pattern of *TLR7* duplication similar to other passerines, or a single copy of *TLR7* as found in non-passerine bird species. In the sequencing data, rock wren showed repeatable variation among individuals in the relative chromatogram peak heights at 12 apparently heterozygous nucleotide sites at *TLR7* ($N = 21$). These observations could be explained by a gene-duplication wherein either copy is heterozygous at these sites. In addition, two individuals exhibited one apparent tri-nucleotide position in the Sanger sequencing chromatograms of two independent PCRs per individual. These two observations support coamplification of a possible gene-duplication of *TLR7* in rock wren, suggesting the duplication occurred early in the divergence of the Passeriformes, although cloning or next-generation sequencing data of the amplified products would be required to confirm this. Due to the possibility of coamplification of orthologous sequences, *TLR7* data for saddleback and rock wren were excluded from subsequent analyses.

Selection on TLR sequences

Within species, three loci showed statistically significant heterozygote excess (kiwi *TLR2B*, takahe *TLR15* and hihi

Table 1 TLR sequencing results and diversity measures

Locus	Species	N^a	bp (aa)[b]	Ch[c]	SNPs (s:n)[d]	h^e	k^f	π^g	Genbank[h]
TLR1LA	Kiwi	18	926 (308)	226	1 (1:0)	2	0.11	0.12	KF265255, KP249944–5
	Takahe	20	896 (298)	227	0 (0:0)	1	–	–	KF265263
	Kakapo	17	1184 (394)	222	1 (1:0)	2	0.17	0.12	KF265264, KP249932
	Kakariki	18	1165 (388)	227	14 (10:4)	8	4.23	3.63	KF265258, KP249933–43
	Rock wren	21	995 (331)	225	15 (7:8)	13	4.08	4.10	KF265265, KP249965–81
	Mohua	21	987 (329)	227	3 (1:2)	4	0.80	0.81	KF265259, KP249950–6
	Robin	22	963 (321)	229	7 (3:4)	7	1.23	1.27	KP249957–64
	Hihi	19	941 (313)	229	7 (4:3)	6	2.50	2.66	KF265260, KP249927–31
	Kokako	21	903 (301)	228	2 (1:1)	3	0.64	0.71	KF265256, KP249946–9
	Saddleback	20	1049 (349)	228	2 (0:2)	3	0.65	0.62	KF265261–2, KP249982–4
	Mean	19.7	1000.9 (333.2)		5.2 (2.8:2.4)	4.9	1.440	1.404	
TLR1LB	Takahe	20	746 (248)	120	0 (0:0)	1	–	–	KF265273
	Kakariki	20	874 (291)	143	21 (14:7)	6	6.68	7.65	KF265267, KP249988–97
	Rock wren	22	968 (322)	120	15 (12:3)	16	3.19	3.29	KF265274, KP250017–34
	Mohua	21	897 (299)	143	3 (3:0)	4	1.27	1.42	KF265269–70, KP250000–5
	Robin	19	835 (278)	143	6 (3:3)	7	1.98	2.37	KP250006–16
	Hihi	20	879 (293)	143	4 (2:2)	4	0.47	0.53	KF265271, KP249985–7
	Kokako	23	880 (293)	144	1 (0:1)	2	0.16	0.18	KF265266, KP249998–9
	Saddleback	20	948 (316)	121	0 (0:0)	1	–	–	KF265272
	Mean	20.6	878.4 (292.5)		6.3 (4.3:2)	5.1	1.719	1.930	
TLR2B	Kiwi	20	997 (332)	232	5 (3:2)	6	1.62	1.62	KF265275–6, KP250035–9
	Kakapo	19	1189 (396)	235	0 (0:0)	1	–	–	KF265281
	Robin	24	936 (312)	283	7 (6:1)	6	1.58	1.69	KP250040–6
	Hihi	19	942 (314)	236	0 (0:0)	1	–	–	KF265279
	Saddleback	19	1023 (341)	236	0 (0:0)	1	–	–	KF265280
	Mean	20.2	1017.4 (339)		2.4 (1.8:0.6)	3.0	0.639	0.662	
TLR3	Kiwi	20	777 (259)	273	2 (0:2)	3	0.19	0.25	KF265282, KP250047–8
	Takahe	19	976 (325)	248	0 (0:0)	1	–	–	KF265288
	Kakapo	18	1121 (373)	253	0 (0:0)	1	–	–	KF265289
	Rock wren	21	947 (315)	245	5 (1:4)	6	0.70	0.74	KF265290, KP250057–63
	Mohua	23	920 (306)	264	1 (0:1)	2	0.39	0.43	KF265285–6, KP250050–1

Table 1 continued

Locus	Species	N^a	bp (aa)[b]	Ch[c]	SNPs (s:n)[d]	h^e	k^f	π^g	Genbank[h]
	Robin	20	902 (300)	260	3 (2:1)	4	0.38	0.42	KP250052–6
	Kokako	19	881 (293)	248	1 (1:0)	2	0.05	0.06	KF265283, KP250049
	Saddleback	19	925 (308)	247	0 (0:0)	1	–	–	KF265287
	Mean	19.9	931.1 (309.9)		1.5 (0.5:1)	2.5	0.215	0.238	
TLR4	Takahe	19	788 (262)	214	1 (0:1)	2	0.10	0.13	KF265298–9
	Kakariki	20	849 (283)	191	8 (2:7)[i]	7	2.39	2.82	KF265291, KP250064–74
	Rock wren	21	704 (234)	213	10 (5:5)	8	2.35	3.33	KF265301, KP250090–101
	Mohua	24	660 (220)	230	2 (1:1)	3	0.97	1.47	KF265293–4, KP250075–77
	Robin	23	635 (211)	232	7 (1:7)[i]	8	1.79	2.83	KP250078–89
	Saddleback	20	647 (215)	232	0 (0:0)	1	–	–	KF265300
	Mean	21.2	713.8 (237.5)		4.7 (1.5:3.5)	4.8	1.267	1.763	
TLR5	Kiwi	19	960 (320)	211	1 (0:1)	2	0.24	0.24	KF265302–3, KP250111
	Takahe	20	892 (297)	241	0 (0:0)	1	–	–	KF265312
	Kakapo	18	897 (299)	258	0 (0:0)	1	–	–	KF265313
	Kakariki	20	961 (320)	262	7 (3:4)	8	2.10	2.19	KF265306, KP250102–10
	Mohua	20	1035 (345)	206	11 (6:5)	6	3.31	3.20	KF265308–9, KP250125–32
	Robin	20	838 (279)	216	2 (0:2)	4	0.64	0.77	KP250133–37
	Kokako	24	863 (287)	207	9 (2:7)	8	2.44	2.83	KF265304–5, KP250112–24
	Saddleback	19	1024 (341)	209	0 (0:0)	1	–	–	KF265311
	Mean	20.0	933.8 (311)		3.8 (1.4:2.4)	3.9	1.092	1.154	
TLR7	Kiwi	20	808 (269)	226	2 (1:1)	2	0.90	1.11	KF265314–5, KP250140
	Kakapo	19	729 (243)	231	0 (0:0)	1	–	–	KF265324
	Kakariki	18	730 (243)	231	1 (1:0)	2	0.46	0.63	KF265318, KP250138–9
	Rock wren	21	885 (295)	214	–[j]	–	–	–	KF265325, KP250141–59
	Saddleback	20	927 (309)	213	–[j]	–	–	–	KF265322
	Mean	19.6	815.8 (271.8)		1 (0.7:0.3)	1.7	0.452	0.580	
TLR15	Takahe	20	987 (329)	163	1 (0:1)	2	0.48	0.49	KP250190–1
	Mohua	21	976 (325)	123	3 (0:3)	4	1.13	1.16	KF265328–9, KP250176–81
	Robin	19	913 (304)	143	5 (2:3)	8	0.90	0.98	KP250182–9
	Hihi	18	975 (325)	135	1 (1:0)	2	0.48	0.49	KF265330–1, KP250160–2
	Kokako	21	993 (331)	137	14 (10:4)	14	1.95	1.96	KF265326–7, KP250163–75
	Saddleback	19	1052 (350)	137	0 (0:0)	1	–	–	KF265332
	Mean	19.7	982.7 (327.3)		4 (2.2:1.8)	5.2	0.823	0.847	

Table 1 continued

Locus	Species	N^a	bp (aa)[b]	Ch[c]	SNPs (s:n)[d]	h^e	k^f	π^g	Genbank[h]
TLR21	Takahe	20	752 (250)	441	0 (0:0)	1	–	–	KF265339
	Mohua	20	641 (213)	463	0 (0:0)	1	–	–	KF265336
	Robin	22	640 (213)	464	3 (2:1)	5	1.11	1.73	KP250195–202
	Hihi[k]	19	628 (209)	464	5 (0:5)	4	0.92	1.46	KF265337–8, KP250192–4
	Kokako	19	605 (201)	474	0 (0:0)	1	–	–	KF265335
	Mean	20.0	653.2 (217.2)		1.6 (0.4:1.2)	2.4	0.405	0.638	
Grand	Maximum	24	1189 (396)		21 (14:8)	16	6.68	7.65	
	Mean	20.1	894.5 (297.8)		3.7 (1.9:1.8)	4	0.98	1.09	
	Minimum	17	605 (201)		0 (0:0)	1	0.00	0	
	Total	1,225	54566 (18,168)		219 (109:112)[i]	233			

[a] Number of individuals included in each alignment

[b] Length of fragment (*bp* base-pairs, *aa* amino acids)

[c] Position of the first amino acid relative to chicken TLRs; Genbank accessions of reference chicken proteins are *TLR1LA*: BAD67422.1, *TLR1LB*: ABF67957.1, *TLR2B*: BAB16842.1, *TLR3*: NP_001011691.3, *TLR4*: AAL49971.1, *TLR5*: CAF25167.1, *TLR7*: NP_001011688.1, *TLR15*: NP_001032924.1, *TLR21*: NP_001025729.1

[d] Number of synonymous (s) or non-synonymous (n) single nucleotide polymorphisms (SNPs)

[e] Number of inferred haplotypes

[f] Mean nucleotide diversity (based on inferred haplotypes)

[g] Mean number of nucleotide differences (based on inferred haplotypes)

[h] Genbank accession numbers of representative sequences from each alignment

[i] Triallelic SNPs observed

[j] Examination of sequencing chromatograms suggested coamplification of duplicate loci

[k] Two individuals, which were apparently heterozygous for a 3-bp indel variant, were excluded from this dataset

Table 2 Summary statistics for microsatellite data

Species	$N^a_{samples}$	N^b_{loci}	A (SD)[c]	H_E (SD)[d]	References
Kiwi	20.8	13	3.77 (1.69)	0.477 (0.237)	Current study
Takahe[e]	22.8	23	2.25 (0.61)	0.405 (0.156)	Grueber et al. (2011)
Kakapo	17.0	25	2.72 (0.89)	0.479 (0.164)	B.R. unpubl. data
Kakariki	19.3	14	3.79 (1.85)	0.454 (0.231)	G.K. unpubl. data
Rock wren	23.0	13	4.54 (1.39)	0.594 (0.192)	Weston (2014)
Mohua	25.0	11	2.70 (1.06)	0.411 (0.172)	Jamieson et al. (2007)
NZ robin	25.0	10	4.00 (2.12)	0.489 (0.266)	Boessenkool et al. (2007)
Hihi	18.7	20	4.80 (2.40)	0.663 (0.186)	P.B. unpubl. data
Kokako	20.9	8	5.25 (2.87)	0.576 (0.245)	Current study
Saddleback	19.7	9	2.33 (0.71)	0.411 (0.131)	Taylor and Jamieson (2008a, b)

[a] Mean number of genotyped individuals across microsatellite loci, all individuals were genotyped at ≥1 polymorphic TLR locus

[b] Number of polymorphic loci

[c] Mean number of alleles (standard deviation)

[d] Mean expected heterozygosity, adjusted for sample size using Levene's correction (Levene 1949) (standard deviation)

[e] For takahe, two published loci (*Pho06* and *Pho38*) were reported as Z-linked (Grueber et al. 2011), so we excluded genotypes of females and unknown sex individuals for these loci

TLR1LA) and one showed significant heterozygote deficit (robin *TLR21*) at $\alpha = 0.05$ (Supplementary Table S2), although only hihi *TLR1LA* was significant after accounting for multiple testing (sequential Bonferroni correction, 41 tests; Holm 1979). Furthermore, slight heterozygote excess was observed at approximately half (49 %) of our 41 gene/

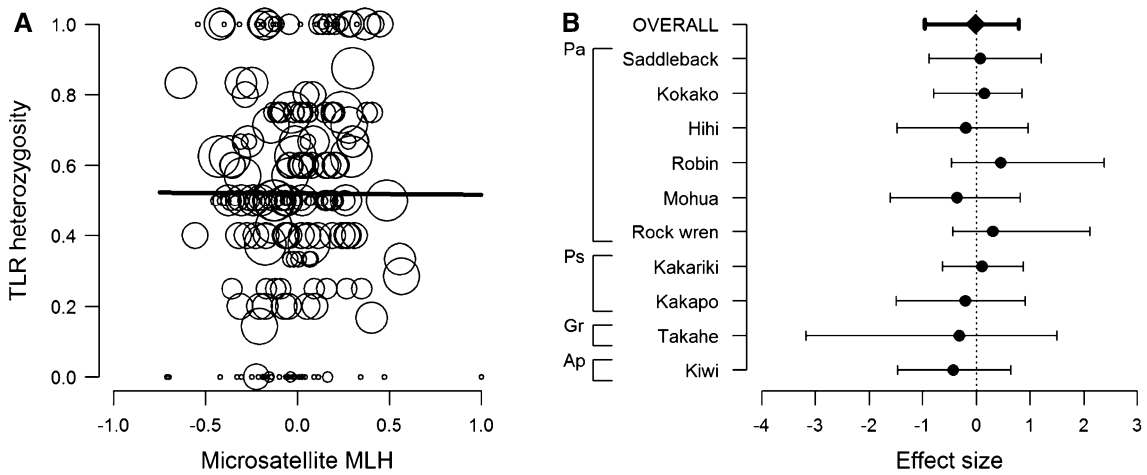

Fig. 2 Relationship between IR, a microsatellite-based measure of individual multilocus heterozygosity (MLH), and TLR heterozygosity. **A** The data from each individual ($N = 216$); the size of each point indicates the number of TLR loci genotyped for that individual (range 1–8). The *fitted line* on **A** is the main effect indicated as "overall" in **B**. **B** Forest plot comparing the species-level slopes on the logit scale (random slopes model fitted using *MCMCglmm*; see "Methods" section). *Points* are the posterior mean; *error bars* are the 95 % credible interval. Species are indicated on the *y*-axis of **B**, grouped by avian order ("Pa" Passeriformes, "Ps" Psittaciformes, "Gr" Gruiformes, "Ap" Apterygiformes)

Table 3 Linear mixed model estimates for TLR heterozygosity

Model factor	Estimate	CI (lower)	CI (upper)
Fixed effects			
Intercept	0.083	−0.355	0.591
Slope (microsatellite IR)	−0.013	−0.957	0.790
Random effects (variance components)			
Intercept (species)	0.340	0.036	1.493
Slope (species)	0.023	0.001	3.435
Covariance (intercept : slope)	0.152	−0.390	2.188
Residual	0.001	1.808×10^{-4}	0.105

Estimates (on the logit scale) are based on means of posterior distributions (modes for random effects) and are presented alongside 95 % credible intervals (CI)

species samples, suggesting no systematic pattern of deviation from Hardy–Weinberg equilibrium at toll-like receptor loci among the 10 species studied here (Supplementary Table S2). Therefore, with the possible exception of hihi *TLR1LA*, there was no strong evidence for balancing selection at TLR loci within these species.

Most Tajima's *D* and Ewens–Watterson test statistics showed no deviations from neutrality for the four species that had ≥ 2 loci with ≥ 2 haplotypes (Supplementary Table S3). These results are consistent with genetic drift as the predominant determinant of the observed haplotype frequencies in these four species. One exception was *TLR5* for kokako, for which the Ewens–Watterson test statistic indicated that allele frequencies were more uniform than predicted under neutrality (Supplementary Table S3). This result suggests that a form of balancing selection is potentially operating on *TLR5* in kokako, an observation that is unlikely to result from demographically induced biases (such as changes in population size or incomplete isolation), as *TLR15* showed no such deviation in kokako and all loci are expected to be similarly impacted by demographic processes (Supplementary Table S3).

Comparing diversity of the three TLRs with viral ligands (*TLR3*, *TLR7* and *TLR21*) to the other TLRs, which have proteinaceous ligands, we observed lower diversity among the virus-sensing loci in terms of h, π, k and number of SNPs (Supplementary Fig. S1). The 95 % confidence intervals for the regression slopes excluded zero for all diversity measures, with viral-sensing TLRs showing, on average, 2.2 fewer haplotypes, k decreased by 0.557, π decreased by 0.00048 and 2.7 fewer SNPs (1.1 fewer synonymous; 1.6 fewer non-synonymous) than TLRs that bind proteinaceous ligands, as inferred from the modelling results (Supplementary Table S3). Together, these results highlight potential differences in long-term (i.e. over evolutionary timescales) selection pressures experienced by different TLR loci.

Association of microsatellite and TLR diversities

Across all 10 study species, we recorded microsatellite genotype diversity data for a total of 146 polymorphic loci, representing a mean of 14.6 loci per species (range 8 [kokako] to 25 [kakapo] loci) (Table 2). We used internal relatedness (IR) as our metric of individual MLH, noting

that IR is a measure of homozygosity, and is expected to decrease with increasing levels of genome-wide heterozygosity (e.g. IR is expected to correlate positively with inbreeding coefficient).

On average across all species, MLH showed no relationship with TLR heterozygosity; the very weak negative effect was statistically non-significant: the 95 % credible interval included zero (Fig. 2A; full model results in Supplementary Table S5). The model suggested that there were some species-specific effects (Fig. 2B): species-level slope variance was 0.023, translating to a between-species standard deviation in slopes of 0.153 (Table 3). Comparing this value to the magnitude of the overall slope (−0.013; Table 3), suggests that there may be some variation among species, however as seen in Fig. 2B, all species display similar null relationships. Note that these values are all estimated with poor precision (Table 3).

We did observe some variation in the precision of slope estimates (i.e. the slope errors; Fig. 2B). It is possible that variation in the number of microsatellite loci used for each species may contribute to this variation, as well as the diversity of those loci (see Table 2). This may occur if, for example, greater numbers of microsatellite loci, or loci with greater diversity, facilitate more-precise estimates of individual MLH than using less-informative microsatellite data. This hypothesis would predict a negative relationship between the amount of microsatellite data and the magnitude of species-level slope errors (width of the 95 % CI for the relationship between microsatellite MLH and TLR heterozygosity of each species). We found no such relationship with the number of loci used, mean gene diversity (expected heterozygosity) of loci, nor mean number of alleles at microsatellite loci (Supplementary Fig. S2). Therefore we had no evidence that the variation in slope errors was correlated with the amount or quality of microsatellite data used.

Discussion

Here we report population-level toll-like receptor diversity statistics for 10 threatened New Zealand bird species. Many loci were variable: SNPs were observed in all sequenced loci for three species, and all species were polymorphic at one or more TLR loci (Table 1).

A recent phylogenetic study of the evolution of TLRs across bird species found a high degree of episodic positive selection, consistent with a pathogen-mediated model of evolution (Grueber et al. 2014). At the within-population level, the current study provided little evidence for balancing selection at TLR loci within these 10 populations. Although hihi appeared to have an excess of heterozygotes at *TLR1LA*, there was no systematic pattern of heterozygote excess (relative to Hardy–Weinberg expectations) across species or genes, indicating no evidence of balancing selection. We note, however, that comparing the frequency of observed heterozygotes to Hardy–Weinberg expectations only permits detection of selection over a single generation; very strong selection would be required to drive a statistically significant result over this short timeframe (Spurgin and Richardson 2010; Hedrick 2012). Comparing haplotype frequencies to neutral expectations for four species also failed to produce evidence of balancing selection, with the possible exception of *TLR5* for kokako. This latter result is surprising, given a recent report of multiple independent *TLR5* pseudogenisation events in passerine evolution (Bainová et al. 2014). It is unclear why these differences among species occur (Bainová et al. 2014), although further investigation into specific pathogen pressures experienced by these species, with specific comparison to kokako, would be valuable. Overall, a general lack of evidence for balancing selection in these small populations supports findings from a pedigree-based study of a population of Stewart Island robin that genetic drift is a key determinant of population TLR diversity following a bottleneck (Grueber et al. 2013). These results are also similar to findings from studies of MHC, which have found that genetic drift can be a strong determinant of diversity after a severe population bottleneck (e.g. Miller and Lambert 2004).

We observed no relationship between microsatellite IR (a measure of homozygosity) and TLR heterozygosity across individuals from 10 threatened New Zealand bird species. These results suggest that microsatellite MLH is not a good indicator of inter-individual variation in heterozygosity at genic regions, such as TLR loci. These findings support claims that microsatellite MLH, estimated with a relatively small number of markers, is often not a reliable predictor of individual-level genome-wide heterozygosity (Balloux et al. 2004; Miller et al. 2014). Importantly, our results highlight the value of studying functional genomic regions, such as TLR sequences, due to the additional information these loci can provide about changes in genetic diversity in conservation contexts.

All of the species we examined are threatened and have therefore undergone population bottlenecking to varying degrees ("Appendix" section). A recent report of population-level TLR diversity in two widespread species, house finch *Carpodacus mexicanus* and lesser kestrel *Falco naumanni*, indicated much higher levels of TLR diversity than we observed in the 10 threatened species studied here (Alcaide and Edwards 2011; Table 1). To examine the association between microsatellite and TLR heterozygosity in the current study, we focused on *within* population relationships (i.e. utilising individual-level statistics). It was not possible to evaluate the relationship between mean microsatellite and TLR diversity *across* populations (i.e. utilising

population-level statistics, such as population size), for two important reasons. First, technical differences in microsatellite characterisation and genotyping protocols between species would likely result in non-homology between loci across species, as well as cross-species variation in ascertainment bias. For example, microsatellite data for some species comprised mainly loci characterised for the species themselves (e.g. takahe; Grueber et al. 2008a), while others primarily used loci characterised in related species (e.g. robin; Boessenkool et al. 2007). Thus, population-level statistics based on microsatellite data cannot be directly compared across species. Second, we were unable to amplify the same TLR loci in all species (although there was a high degree of overlap, Table 1). TLRs bind a diversity of pathogen-associated molecular patterns, so amplifying different loci across species may drive differences in mean levels of diversity. For example, we observed reduced TLR diversity in viral-sensing TLRs, compared to other TLRs (Supplementary Fig. S1), similar to findings in mammals (Areal et al. 2011). Furthermore, even when considering the same TLR locus, differences in evolutionary, ecological and life-history traits of each species may drive differences in selection on TLR diversity, complicating comparisons across species. We do not have data regarding the particular parasite or disease burdens experienced by any of the individuals included herein, which might have enabled us to partially control for these effects.

Overall, we have observed that, within populations of conservation concern, microsatellite MLH contains no signal of inter-individual variation in heterozygosity of TLRs. Thus, because of their ease of genotyping (relative to MHC immunity genes), TLRs represent a valuable addition to the conservation genetic toolkit for the study of functional genetic variation in non-model species. The population-level TLR diversity data presented here, for 10 bird species of conservation concern, will provide a valuable comparison for similar studies in common species, as well as a starting point for studies of the relationship between TLR diversity and fitness in these and related taxa.

Acknowledgments We are grateful to those who generously provided samples and support for this study: Hugh Robertson, Oliver Overdyck, Tertia Thurley (New Zealand Department of Conservation); Kevin Parker (Massey University), John Ewen (Zoological Society of London) and Bethany Jackson (Auckland Zoo). We also thank Fiona Robertson for laboratory assistance and Ken Miller for preparation of Fig. 1. We are grateful for the continuing support of the New Zealand Department of Conservation, and in particular those Species Recovery Groups included in this study. This research was supported by the Allan Wilson Centre for Molecular Ecology and Evolution, the Marsden Fund, Landcare Research, University of Otago, a Royal Society Grant to PB and grants from Brian Mason Scientific and Technical Trust, Mohua Charitable Trust and JS Watson Conservation Trust to KW. CEG is currently supported by San Diego Zoo Global.

Appendix

Here we provide species histories and details of study populations for the 10 threatened New Zealand native birds examined in this study. Polynesians were the first settlers to New Zealand from ~1200 AD (Higham et al. 1999), followed by a second colonisation by Europeans in the 1800s. Both immigration events brought about significant environmental changes, and much of New Zealand's vertebrate fauna has been impacted. For example, New Zealand endemic birds have experienced dramatic population bottlenecks as a result of predation by introduced mammals (such as rats *Rattus* sp. and mustelids; New Zealand has no native terrestrial mammals except three species of bat), competition from introduced browsers (such as possums *Trichosurus vulpecula*), and habitat loss and fragmentation due to land conversion (Duncan and Blackburn 2004). These bottlenecks may have resulted in losses of genetic diversity, both at the population level (low numbers of alleles) and at the individual level (low observed heterozygosity). Sites mentioned herein are shown on the map in Fig. 1.

Apterygiformes: North Island brown kiwi *Apteryx mantelli*

The North Island brown kiwi is the most common of the five species of kiwi, currently numbering ~25,000, spread over four genetically distinct forms (Holzapfel et al. 2009). Despite intensive management, the species is classified under the New Zealand threat classification system as in "serious decline", primarily as a result of human induced impacts (habitat loss and introduced mammalian predators, especially stoats *Mustela erminea*), and poor recruitment (Holzapfel et al. 2009; Ziesemann et al. 2011). We used feather samples from a population of brown kiwi near Purua, in the north of the North Island (currently numbers ~8,000; Holzapfel et al. 2009), collected as part of ongoing management activities (H. Robertson pers. comm.).

Photo: Colin Miskelly, NZ Birds online (www.nzbirdsonline.org)

Gruiformes: takahe *Porphyrio hochstetteri*

Takahe were once widespread throughout the South Island of New Zealand, but are thought to have experienced dramatic declines since the arrival of Polynesian settlers in the 1600s and were even thought to be extinct by the 1900s (Lee and Jamieson 2001). A remnant population of birds was discovered in 1948 in the Murchison Mountains, Fiordland and has been subject to intense management since. The current population of <300 comprises birds in the original Murchison Mountains habitat, as well as translocated populations in several predator-free sanctuaries around the country (Wickes et al. 2009). We used samples from the source population, in Fiordland National Park, which were collected for a previous study (Lettink et al. 2002). Previous microsatellite analysis of takahe found very low levels of diversity, both in terms of numbers of polymorphic loci and numbers of alleles at variable loci (Grueber et al. 2008a), probably resulting from a prolonged population bottleneck (Grueber and Jamieson 2011).

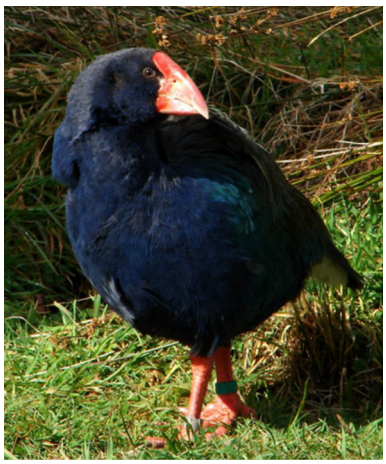

Photo: Catherine Grueber

Psittaciformes: kakapo *Strigops habroptilus*

The kakapo is a large flightless bird, endemic to New Zealand and unique among parrots in its nocturnal, lek-breeding behaviours (Powlesland et al. 2006). The species is severely threatened by the introduction of stoats and rats and were the focus of possibly the earliest-known New Zealand conservation efforts, beginning in 1894. Sadly, only one Fiordland bird ("Richard Henry") was ultimately rescued and translocated to Codfish Island along with members of a remnant population of birds from Stewart Island (Clout 2006). Very low levels of genetic diversity have been observed in kakapo with microsatellites (Robertson et al. 2000). All samples used in this study were collected as part of on-going management and are from Stewart Island-origin birds, i.e. we did not include samples from "Richard Henry" or his offspring. All extant kakapo ($N \sim 124$), except for Richard Henry's offspring, descend from 62 Stewart Island birds (Powlesland et al. 2006).

Photo: Ian Jamieson

Psittaciformes: kakariki (red-crowned parakeet) *Cyanoramphus novaezelandiae*

Kakariki were once abundant on New Zealand's North and South Islands until the introduction of predatory mammals and the destruction of suitable habitat significantly decreased their range (Higgins 1999). Kakariki are now considered effectively extinct from the mainland and only exist on Stewart Island and a number of offshore islands (BirdLife International 2013). We used samples collected from the wildlife sanctuary of Tiritiri Matangi in 2011 as part of concurrent research (B. Jackson pers. comm.). Since the introduction of approximately 90 birds to Tiritiri Matangi Island between 1974 and 1976 (Dawe 1979) and the subsequent eradication of kiore (Pacific rat *Rattus exulans*) from the island (Veitch 2002), the abundance of kakariki at the site has increased considerably.

Photo: Emily Weiser

Passeriformes: New Zealand rock wren *Xenicus gilviventris*

New Zealand rock wren are New Zealand's only true alpine bird, living above the tree line for its entire life (Michelsen-Heath and Gaze 2007). Rock wren have been reported throughout much of the "main divide" of the Southern Alps, although a distribution study based on records of sightings over the past 100 years indicated that the species is declining (Michelsen-Heath and Gaze 2007). The samples we used here were collected from Fiordland, near the Homer Tunnel, as part of on-going research (Weston and Robertson 2014). This location is considered a stronghold for rock wren (Michelsen-Heath and Gaze 2007).

Photo: Bruce Robertson

Passeriformes: mohua (yellowhead) *Mohoua ochrocephala*

Mohua were formerly distributed across the entire South Island of New Zealand, but as a result of land conversion the species became fragmented into eight major forest patches (Gaze 1985; O'Donnell et al. 2002; Tracy and Jamieson 2011). Mohua are further threatened today by introduced mammalian predators, especially stoats (O'Donnell et al. 2002). Microsatellite-based studies of population structure and historical diversity (using museum specimens) revealed that the species has lost a significant amount of allelic diversity over the last 100 years and that a pattern of isolation by distance exists among contemporary mohua populations (Tracy and Jamieson 2011). We used samples from the Dart River Valley, Fiordland, for which DNA had been collected for a previous analysis (Tracy and Jamieson 2011); birds from this site are connected with those from other sites within a large forest patch.

Photo: Scott Mouat

Passeriformes: South Island robin *Petroica australis australis*

The South Island robin is a charismatic forest species endemic to New Zealand, belonging to the widespread Australasian family Petroicidae (Higgins and Peter 2002). Robins were previously distributed throughout the South Island, but the population has been increasingly fragmented as a result of habitat loss. Fragmentation is exacerbated by a reluctance of the birds to cross open water or unforested habitat, further isolating sub-populations of the species. A study of robin microsatellite diversity, in comparison to museum specimens, found little loss of diversity among contemporary birds as a whole, although birds from large mainland populations harboured more diversity than island birds (Boessenkool et al. 2007; Taylor et al. 2007). Here we use samples from one of the largest remnant robin populations, in the Eglinton River Valley, which were collected for a previous study (Boessenkool et al. 2007).

Photo: Ian Jamieson

Passeriformes: hihi (stitchbird) *Notiomystis cincta*

Once distributed throughout the North Island mainland and on northern offshore islands, hihi were extirpated from the mainland by the 1880s and persisted in a single remnant population on Little Barrier Island (Taylor et al. 2005). The Little Barrier Island population has been used as a source for subsequent translocations to establish populations on additional offshore islands (Taylor et al. 2005; Brekke et al. 2011). Microsatellite data revealed that the Little Barrier Island population has relatively high genetic diversity, possibly as a result of a high degree of extra-pair paternity in the species, reducing male reproductive variance (Brekke et al. 2011). We used samples from Little Barrier Island that were collected as part of on-going research (Brekke et al. 2011).

Photo: Paul Gibson

Passeriformes: North Island kokako *Callaeas wilsoni*

Kokako were formerly distributed across the North Island, but as a result of land conversion have become restricted to ~15 isolated forest fragments and introduced to several islands (Innes et al. 1999). The birds are particularly vulnerable to ship rats (*Rattus rattus*) and brushtail possums (*Trichosurus vulpecula*), so the recovery of kokako depends on the management of these invasive species (Basse et al. 2003). Microsatellite data have shown that the three largest kokako populations show only low levels of genetic differentiation (Hudson et al. 2000). Our samples were collected from the Mapara population, as part of ongoing management (O. Overdyck and T. Thurley, pers. comm.). Although the Mapara population is known to have declined to a small number of breeders in the 1990s, its recovery was rapid (Hudson et al. 2000).

Photo: Emily Weiser

Passeriformes: South Island saddleback *Philesturnus carunculatus rufusator*

South Island saddleback underwent an extreme population bottleneck when they were extirpated from the South Island of New Zealand at the time of human settlement and as a result of predation by invasive rats. Saddlebacks suffered a further severe bottleneck in the 1960s when the last known population, on Big South Cape Island, was reduced from around 1,000 birds to just 36 after rats arrived on the island. In 1964, these remaining birds were then moved to two rat-free sites, Big and Kaimohu Islands—the first threatened species translocation carried out by the New Zealand government through its Wildlife Service (later renamed the Department of Conservation) (Merton 1975). These populations grew and have been the source for several subsequent saddleback populations; the total population now numbers around 1,200 (Hoosen and Jamieson 2003). We used samples collected from Big Island as part of a previous study (Taylor and Jamieson 2008a, b).

Photo: Scott Mouat

References

Acevedo-Whitehouse K, Cunningham AA (2006) Is MHC enough for understanding wildlife immunogenetics? Trends Ecol Evol 21:433–438

Alcaide M, Edwards SV (2011) Molecular evolution of the toll-like receptor multigene family in birds. Mol Biol Evol 28:1703–1715

Alho JS, Lillandt BG, Jaari S, Merila J (2009) Multilocus heterozygosity and inbreeding in the Siberian jay. Conserv Genet 10:605–609. doi:10.1007/s10592-008-9588-z

Alho JS, Välimäki K, Merilä J (2010) Rhh: an R extension for estimating multilocus heterozygosity and heterozygosity–heterozygosity correlation. Mol Ecol Resour 10:720–722. doi:10.1111/j.1755-0998.2010.02830.x

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

Amos W, Worthington Wilmer J, Fullard K et al (2001) The influence of parental relatedness on reproductive success. Proc R Soc Lond Ser B 268:2021–2027

Areal H, Abrantes J, Esteves P (2011) Signatures of positive selection in Toll-like receptor (TLR) genes in mammals. BMC Evol Biol 11:368. doi:10.1186/1471-2148-11-368

Babik W, Taberlet P, Ejsmond MJ, Radwan J (2009) New generation sequencers as a tool for genotyping of highly polymorphic multilocus MHC system. Mol Ecol Resour 9:713–719. doi:10.1111/j.1755-0998.2009.02622.x

Bainová H, Králová T, Bryjová A et al (2014) First evidence of independent pseudogenization of Toll-like receptor 5 in passerine birds. Dev Comp Immunol 45:151–155

Balloux F, Amos W, Coulson T (2004) Does heterozygosity estimate inbreeding in real populations? Mol Ecol 13:3021–3031

Barreiro LB, Ben-Ali M, Quach H et al (2009) Evolutionary dynamics of human Toll-like receptors and their different contributions to host defense. PLoS Genet 5:e1000562. doi:10.1371/journal.pgen.1000562

Basse B, Flux I, Innes J (2003) Recovery and maintenance of North Island kokako (*Callaeas cinerea wilsoni*) populations through pulsed pest control. Biol Conserv 109:259–270. doi:10.1016/S0006-3207(02)00154-4

Bates D, Maechler M (2009) lme4: linear mixed-effects models using S4 classes

BirdLife International (2013) *Cyanoramphus novaezelandiae*. IUCN 2013 IUCN Red List Threat. Species Version 20132. BirdLife International, Cambridge

Boessenkool S, Taylor SS, Tepolt CK et al (2007) Large mainland populations of South Island robins retain greater genetic diversity than offshore island refuges. Conserv Genet 8:705–714

Bollmer JL, Dunn PO, Whittingham LA, Wimpee C (2010) Extensive MHC class II B gene duplication in a Passerine, the common yellowthroat (*Geothlypis trichas*). J Hered 101:448–460. doi:10.1093/jhered/esq018

Bollmer JL, Ruder EA, Johnson JA et al (2011) Drift and selection influence geographic variation at immune loci of prairie-chickens. Mol Ecol 20:4695–4706. doi:10.1111/j.1365-294X.2011.05319.x

Brekke P, Bennett PM, Santure AW, Ewen JG (2011) High genetic diversity in the remnant island population of hihi and the genetic consequences of re-introduction. Mol Ecol 20:29–45. doi:10.1111/j.1365-294X.2010.04923.x

Casquet J, Thebaud C, Gillespie RG (2012) Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. Mol Ecol Resour 12:136–141. doi:10.1111/j.1755-0998.2011.03073.x

Chapman JR, Nakagawa S, Coltman DW et al (2009) A quantitative review of heterozygosity–fitness correlations in animal populations. Mol Ecol 18:2746–2765. doi:10.1111/j.1365-294X.2009.04247.x

Clout MN (2006) A celebration of kakapo: progress in the conservation of an enigmatic parrot. Notornis 53:1–2

Coltman DW, Pilkington JG, Smith JA, Pemberton J (1999) Parasite-mediated selection against inbred Soay sheep in a free-living island population. Evolution 53:1259–1267

Cormican P, Lloyd AT, Downing T et al (2009) The avian toll-like receptor pathway—subtle differences amidst general conformity. Dev Comp Immunol 33:967–973. doi:10.1016/j.dci.2009.04.001

Dawe MR (1979) Behaviour and ecology of the red-crowned kakariki (*Cyanoramphus novaezelandiae*) in relation to management. Department of Zoology, University of Auckland, Auckland

Deakin JE (2012) Marsupial genome sequences: providing insight into evolution and disease. Scientifica. doi:10.6064/2012/543176

Duncan RP, Blackburn TM (2004) Extinction and endemism in the New Zealand avifauna. Glob Ecol Biogeogr 13:509–517

Ericson PGP, Christidis L, Cooper A et al (2002) A Gondwanan origin of passerine birds supported by DNA sequences of the endemic New Zealand wrens. Proc R Soc Lond B 269:235–241. doi:10.1098/rspb.2001.1877

Ewens WJ (1972) The sampling theory of selectively neutral alleles. Theor Popul Biol 3:87–112

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564–567

Galan M, Guivier E, Caraux G et al (2010) A 454 multiplex sequencing method for rapid and reliable genotyping of highly polymorphic genes in large-scale studies. BMC Genom 11:296

Garrigan D, Hedrick PW (2003) Perspective: detecting adaptive molecular polymorphism: lessons from the MHC. Evolution 57:1707–1722. doi:10.1111/j.0014-3820.2003.tb00580.x

Gaze PD (1985) Distribution of yellowheads (*Mohoua ochrocephala*). Notornis 32:261–269

Gelman A, Su Y-S, Yajima M, et al (2009) arm: data analysis using regression and multilevel/hierarchical models. R-package. Available at http://CRAN.R-project.org/package=arm

Grueber CE, Jamieson IG (2011) Low genetic diversity and small population size of Takahe *Porphyrio hochstetteri* upon European arrival in New Zealand. Ibis 153:384–394

Grueber CE, Jamieson IG (2013) Primers for amplification of innate immunity Toll-like receptors in threatened birds of the Apterygiformes, Gruiformes, Psittaciformes and Passeriformes. Conserv Genet Resour 5:1043–1047. doi:10.1007/s12686-013-9965-x

Grueber CE, King TM, Waters JM, Jamieson IG (2008a) Isolation and characterization of microsatellite loci from the endangered New Zealand takahe (Gruiformes; Rallidae; Porphyrio hochstetteri). Mol Ecol Resour 8:884–886. doi:10.1111/j.1755-0998.2008.02098.x

Grueber CE, Wallis GP, Jamieson IG (2008b) Heterozygosity–fitness correlations and their relevance to studies on inbreeding depression in threatened species. Mol Ecol 17:3978–3984. doi:10.1111/j.1365-294X.2008.03910.x

Grueber CE, Waters JM, Jamieson IG (2011) The imprecision of heterozygosity–fitness correlations hinders the detection of inbreeding and inbreeding depression in a threatened species. Mol Ecol 20:67–79

Grueber CE, Wallis GP, King T, Jamieson IG (2012) Variation at innate immunity Toll-like receptor genes in a bottlenecked population of a New Zealand robin. PLoS One 7:e45011. doi:10.1371/journal.pone.0045011

Grueber CE, Wallis GP, Jamieson IG (2013) Genetic drift outweighs natural selection at toll-like receptor (TLR) immunity loci in a reintroduced population of a threatened species. Mol Ecol 22:4470–4482. doi:10.1111/mec.12404

Grueber CE, Wallis GP, Jamieson IG (2014) Episodic positive selection in the evolution of avian Toll-like receptor innate immunity genes. PLoS One. doi:10.1371/journal.pone.0089632

Hadfield JD (2010) MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package. J Stat Softw 33:1–22

Hartmann SA, Schaefer HM, Segelbacher G (2014) Genetic depletion at adaptive but not neutral loci in an endangered bird species. Mol Ecol. doi:10.1111/mec.12975

Hedrick PW (1998) Balancing selection and MHC. Genetica 104:207–214

Hedrick PW (2012) What is the evidence for heterozygote advantage selection? Trends Ecol Evol 27:698–704. doi:10.1016/j.tree.2012.08.012

Higgins PJ (1999) Handbook of Australian, New Zealand and Antarctic birds. Oxford University Press, Melbourne

Higgins PJ, Peter JM (2002) Handbook of Australian, New Zealand and Antarctic birds. Volume 6: Pardalotes to shrike-thrushes. Oxford University Press, Melbourne

Higham T, Anderson A, Jacomb C (1999) Dating the first New Zealanders: the chronology of Wairau Bar. Antiquity 73:420–427

Holm S (1979) A simple sequentially rejective multiple test procedure. Scand J Stat 6:65–70

Holzapfel S, Robertson HA, McLennan JA et al (2009) Kiwi (Apteryx spp.) recovery plan, 2008–2018. Department of Conservation, Wellington

Hoosen S, Jamieson IG (2003) The distribution and current status of New Zealand saddleback *Philesturnus carunculatus*. Bird Conserv Int 13:79–95

Hudson QJ, Wilkins JR, Waas JR, Hogg ID (2000) Low genetic variability in small populations of New Zealand kokako *Callaeas cinera wilsoni*. Biol Conserv 96:105–112

Innes J, Hay R, Flux I et al (1999) Successful recovery of North Island kokako *Callaeas cinerea wilsoni* populations, by adaptive management. Biol Conserv 87:201–214. doi:10.1016/S0006-3207(98)00053-6

Jamieson IG, Tracy LN, Fletcher D, Armstrong DP (2007) Moderate inbreeding depression in a reintroduced population of North Island robins. Anim Conserv 10:95–102. doi:10.1111/j.1469-1795.2006.00078.x

Jepson A, Banya W, Sisay-Joof F et al (1997) Quantification of the relative contribution of major histocompatibility complex (MHC) and non-MHC genes to human immune responses to foreign antigens. Infect Immun 65:872–876

Keestra AM, de Zoete MR, Bouwman LI et al (2013) Unique features of chicken Toll-like receptors. Dev Comp Immunol 41:316–323. doi:10.1016/j.dci.2013.04.009

Keller LF, Waller DM (2002) Inbreeding effects in wild populations. Trends Ecol Evol 17:230–241. doi:10.1016/S0169-5347(02)02489-8

Kirk H, Freeland JR (2011) Applications and implications of neutral versus non-neutral markers in molecular ecology. Int J Mol Sci 12:3966–3988

Lee WG, Jamieson IG (2001) The takahe: fifty years of conservation management and research. University of Otago Press, Dunedin

Lettink M, Jamieson IG, Millar CD, Lambert DM (2002) Mating system and genetic variation in the endangered New Zealand takahe. Conserv Genet 3:427–434

Levene H (1949) On a matching problem arising in genetics. Ann Math Stat 20:91–94. doi:10.1214/aoms/1177730093

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452. doi:10.1093/bioinformatics/btp187

Ljungqvist M, Akesson M, Hansson B et al (2010) Do microsatellites reflect genome-wide genetic diversity in natural populations? A comment on Väli et al. (2008). Mol Ecol 19:851–855

Meglécz E, Piry S, Desmarais E et al (2011) SESAME (SEquence Sorter & AMplicon Explorer): genotyping based on high-throughput multiplex amplicon sequencing. Bioinformatics 27:277–278. doi:10.1093/bioinformatics/btq641

Merton DV (1975) The saddleback: its status and conservation. In: Martin RD (ed) Breeding endangered species in captivity. Academic Press, London, pp 61–74

Michelsen-Heath S, Gaze P (2007) Changes in abundance and distribution of the rock wren (*Xenicus gilviventris*) in the South Island, New Zealand. Notornis 54:71–78

Miller HC, Lambert DM (2004) Genetic drift outweighs balancing selection in shaping post-bottleneck major histocompatibility complex variation in New Zealand robins (Petroicidae). Mol Ecol 13:3709–3721

Miller JM, Malenfant RM, David P et al (2014) Estimating genome-wide heterozygosity: effects of demographic history and marker type. Heredity 112:240–247. doi:10.1038/hdy.2013.99

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York

O'Donnell CFJ, Roberts A, Lyall J (2002) Mohua (yellowhead) recovery plan 2002–2012. Department of Conservation, Wellington

Piertney SB, Oliver MK (2006) The evolutionary ecology of the major histocompatibility complex. Heredity 96:7–21. doi:10.1038/sj.hdy.6800724

Powlesland RG, Merton DV, Cockrem JF (2006) A parrot apart: the natural history of the kakapo (*Strigops habroptilus*), and the context of its conservation management. Notornis 53:3–26

R Core Team (2013) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Robertson BC, Minot EO, Lambert DM (2000) Microsatellite primers for the kakapo (*Strigops habroptilus*) and their utility in other parrots. Conserv Genet 1:93–95

Seutin G, White BN, Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. Can J Zool 69:82–90

Slate J, David P, Dodds KG et al (2004) Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: theoretical expectations and empirical data. Heredity 93:255–265

Spurgin LG, Richardson DS (2010) How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. Proc R Soc B Biol Sci 277:979–988

Szulkin M, Bierne N, David P (2010) Heterozygosity–fitness correlations: a time for reappraisal. Evolution 64:1202–1217

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595

Taylor SS, Jamieson IG (2008a) No evidence for loss of genetic variation following sequential translocations in extant populations of a genetically depauperate species. Mol Ecol 17:545–556. doi:10.1111/j.1365-294X.2007.03591.x

Taylor SS, Jamieson IG (2008b) No evidence for loss of genetic variation following sequential translocations in extant populations of a genetically depauperate species. Mol Ecol 17:545–556. doi:10.1111/j.1365-294X.2007.03591.x

Taylor C, Castro IC, Griffiths R (2005) Hihi/stitchbird (*Notiomystis cincta*) recovery plan, 2004–2009. Department of Conservation, Wellington

Taylor SS, Jamieson IG, Wallis GP (2007) Historic and contemporary levels of genetic variation in two New Zealand passerines with different histories of decline. J Evol Biol 20:2035–2047

Temperley N, Berlin S, Paton I et al (2008) Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. BMC Genomics 9:62

Tracy L, Jamieson I (2011) Historic DNA reveals contemporary population structure results from anthropogenic effects, not pre-fragmentation patterns. Conserv Genet 12:517–526. doi:10.1007/s10592-010-0158-9

Turner AK, Begon M, Jackson JA et al (2011) Genetic diversity in cytokines associated with immune variation and resistance to multiple pathogens in a natural rodent population. PLoS Genet 7:e1002343

Uematsu S, Akira S (2008) Toll-like receptors (TLRs) and their ligands. Handb Exp Pharmacol 183:1–20. doi:10.1007/978-3-540-72167-3_1

Väli U, Einarsson A, Waits L, Ellegren H (2008) To what extent do microsatellite markers reflect genome-wide genetic diversity in natural populations? Mol Ecol 17:3808–3817. doi:10.1111/j.1365-294X.2008.03876.x

Veitch CR (2002) Eradication of Pacific rats (*Rattus exulans*) from Tiritiri Matangi Island, Hauraki Gulf, New Zealand. In: Veitch CR, Clout MN (eds) Turning the tide: the eradication of invasive species. IUCN, Gland, pp 360–364

Vinkler M, Albrecht T (2009) The question waiting to be asked: innate immunity receptors in the perspective of zoological research. Folia Zool 58:15–28

Walsh PS, Metzger DA, Higuchi R (1991) Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10:506–513

Watterson GA (1978) An analysis of multi-allelic data. Genetics 88:171–179

Werling D, Jann OC, Offord V et al (2009) Variation matters: TLR structure and species-specific pathogen recognition. Trends Immunol 30:124–130. doi:10.1016/j.it.2008.12.001

Westerdahl H (2007) Passerine MHC: genetic variation and disease resistance in the wild. J Ornithol 148:469–477. doi:10.1007/s10336-007-0230-5

Weston KA (2014) Conservation genetics of rock wren (*Xenicus gilviventris*). PhD, Department of Zoology, University of Otago

Weston KA, Robertson BC (2014) Isolation and characterisation of 14 microsatellites for the New Zealand rock wren *Xenicus gilviventris*. Conserv Genet Resour. doi:10.1007/s12686-013-0018-2

Wickes C, Crouchley D, Maxwell J (2009) Takahe recovery plan, 2007–2012. Department of Conservation, Wellington

Ziesemann B, Brunton DH, Castro IC (2011) Nesting success and breeding ecology in a high-density population of Brown Kiwi (*Apteryx mantelli*). Emu 111:148–154