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TECHNICAL NOTE

Primers for amplification of innate immunity toll-like receptor loci in threatened birds of the Apterygiformes, Gruiformes, Psittaciformes and Passeriformes

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Abstract Assaying diversity at functional genomic regions, such as those of the immune system, allows us to test hypotheses about processes that determine the distribution of genetic diversity in threatened populations, and the fitness consequences of those distributions. Toll-like receptors (TLR) are a family of genes responsible for initiating innate and acquired immune responses to a diversity of pathogens. We provide 30 new primers, which, along with cross-species application of published primers, amplify TLR gene sequences in nine bird species of conservation concern in New Zealand. By including one member each of Apterygiformes and Gruiformes, two members of Psittaciformes, and five members of Passeriformes, our data significantly expand the number of avian species for which TLR sequences are available, and facilitates study of these genes in a greater diversity of taxa.

Keywords *Apteryx* · *Callaeas* · *Cyanoramphus* · Evolution · Immunogenetics · *Mohoua* · *Notiomystis* · *Philesturnus* · *Porphyrio* · *Strigops* · *Xenicus*

Immune genes are ideal for studying the evolutionary processes affecting genetic diversity of wild populations, as they represent the most rapidly evolving genes within the genome, due to selection pressure from a wide diversity of coevolving pathogens (Hedrick 1998; Piertney and Oliver 2006). While genes of the major-histocompatibility complex have been widely used for assaying levels of functional diversity, variation at other immunity genes also affects variation in individual immune responses, and are thus of interest to population geneticists (Acevedo-Whitehouse and Cunningham 2006; Vinkler and Albrecht 2009; Turner et al. 2012). One such gene family is toll-like receptors (TLRs), which recognise a wide diversity of pathogens and are responsible for initiating the innate and acquired immune responses (Uematsu and Akira 2008). TLR sequence variation in wild populations has been associated with variation in resilience to infections (Villaseñor-Cardoso and Ortega 2011), and may influence variation in survival of wild animals (e.g. Grueber et al. in press). TLRs also offer some technical advantages over molecular genotyping of MHC (Grueber et al. 2012). Most previous studies of avian TLR diversity have primarily focussed on common or domestic species (Alcaide and Edwards 2011). Expanding the conservation genetics toolkit by examining genic regions associated with a variety of crucial biological processes, including those of the immune system, can provide valuable data for effective management of wild populations (Acevedo-Whitehouse and Cunningham 2006). We aim to provide additional genetic resources (PCR primers and sequences) to facilitate the examination of TLR diversity in threatened birds.

Our study species included one member of Apterygiformes (North Island brown kiwi *Apteryx mantelli*), one member of Gruiformes (takahe *Porphyrio hochstetteri*), two members of Psittaciformes (kakariki [red-crowned parakeet] *Cyanoramphus novaezelandiae* and kakapo *Strigops habroptilus*) and five members of Passeriformes (kokako *Callaeas wilsoni*, hihi [stitchbird] *Notiomystis cincta*, mohua [yellowhead] *Mohoua ochrocephala*, South Island saddleback *Philesturnus carunculatus* and New Zealand rock

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Table 1 Sequences of PCR primers developed in this study

Target locus	Primer	Sequence (5′–3′)
TLR1LB	PcaTLR1LBF	TCAGACTGTATGGCACTCATCC
	PcaTLR1LBR	TCAGCAGCAGAGCTGTCAC
TLR3	DnoTLR3F	CTCCCTGAGCCATGTGAAG
	DnoTLR3R	ATGTTTCCAAAGCCGAGCTA
	PcaTLR3F	CGATYCGGAACCTCTCAC
	PcaTLR3R	TCTGAAGGTTCAATGAATTYAGAGAG
TLR4	AplTLR4R	CCTGCCATCTTGAGCACTTG
	MunTLR4F	CAGCAACCTGACCTCTCTCAG
	MunTLR4R	GCCTGCCATCTTCAGCAC
	PassTLR4F	TCCAGAGGAGACCTTGATGC
TLR5	AalTLR5R	CATTCAGCTACACTAGCAGGCTCT
	OleTLR5F	CCAAATGCCCAAATCCTTTC
	PassTLR5F	AAGTGCCCCAATCCTTTC
	PpoTLR5R	GTGGGAAAAGCCCAGGAG
	ShaTLR5F	AAGTCCAAGCTTTCTCTTCAGC
	ShaTLR5R	TCATAAAGACTTCAGGCTCAGG
TLR7	AalTLR7F	ACAATATTATCCCTGAAGTCTAACAAC
	AalTLR7R	AGCAGCCTCTTTGTCTTTGG
	DnoTLR7F	TCTGGACCTGAAAAATTTAACAATAC
	DnoTLR7R	CAAGCAATCCTCATTAACCAAAG
	parrotTLR7F	ATAACAAGATTCAAGTGATTCAAGAAC
	parrotTLR7R	TACTTGCCTGTTRTATTGCTCTACTG
	PcaTLR7R	ACATTGTTTCTGCTTAAATCC
TLR15	finchTLR15F	GATCTCCCATCCCACCTGA
	FnaTLR15R	GGCAGTTCTGAGATGAGGTTG
	PpoTLR15F	GATCGCCAGAGGCTGCTC
TLR21	CciTLR21F	TCAACCTGCGCAATAACC
	CciTLR21R	CAGCTCCTTCAGTCGTGTCA
	PpoTLR21F	TGTTGCGCCTCGACGTCA
	PpoTLR21R	CAGGTAGCGCAGGGCTGG

wren *Xenicus gilviventris*). Samples were collected for previous (Grueber et al. 2008; Taylor and Jamieson 2008; Tracy and Jamieson 2011) or concurrent studies and were provided to us either as extracted gDNA, whole blood in ethanol or lysis buffer (Seutin et al. 1991), or feathers. We used two samples from all species (except one from *X. gilviventris*) as an internal control against sample handling errors. Where tissue samples were provided, DNA was purified using a modified Chelex (Bio-Rad) extraction [based on Walsh et al. (1991), Casquet et al. (2012)] (blood samples), followed by an additional ethanol precipitation using GenElute linearised polyacrylamide (Sigma) as a DNA carrier (feather samples).

We first attempted to amplify TLR sequences in all species using published primers (Alcaide and Edwards 2011; Grueber et al. 2012). If these primers failed to produce a clear product, we re-examined the avian alignment for the locus in question (Alcaide and Edwards 2011). Primers were then designed at conserved regions using Primer3 (Rozen and Skaletsky 1998); ambiguities in the alignment were resolved to the most taxonomically similar species for which sequence was available (either published sequences available on Genbank, or sequences obtained herein). Our priority was to amplify fragments of maximal length, so we only designed internal primers in cases where no product amplified, or the sequence data was of too low quality to reliably identify ambiguities. Where multiple primer pairs were successful, we report only the pair that provides that longest fragment.

Amplification, clean up and sequencing followed Grueber et al. (2012). Amplifications were performed in a total volume of 15 µl, containing 1 µl extracted DNA, 0.6 U MyTaq polymerase (Bioline Ltd), 1× MyTaq buffer (giving a final concentration of 3 mM Mg^{2+} and 1 mM dNTPs) and 500 nM each primer (Sigma-Aldrich). Thermocyling conditions (on an Eppendorf Mastercycler pro S) consisted of 94 °C for 3 min, followed by 35 cycles of 94 °C for 40 s, locus-specific annealing temperature (Alcaide and Edwards

Table 2 Primers and annealing temperatures (Ta) used to amplify partial Toll-like receptor sequences in nine threatened New Zealand bird species (lengths shown are total amplicon length, including primers, and are approximate)

Species and locus	Forward	Reverse	Ta	Length (bp)	Genbank accession numbers[d]
Apterygiformes: *Apteryx mantelli*					
TLR1LA	avTLR1LAF[a]	avTLR1LAR[a]	58	1,267	KF265255
TLR2B	avTLR2F[a]	avTLR2R[a]	50	1,285	KF265275-6
TLR3	DnoTLR3F[b]	DnoTLR3R[b]	56	856	KF265282
TLR5	avTLR5F[a]	avTLR5R[a]	50	1,345	KF265302-3
TLR7	DnoTLR7F[b]	DnoTLR7R[b]	56	883	KF265314-5
Gruiformes: *Porphyrio hochstetteri*					
TLR1LA	avTLR1LAF[a]	avTLR1LAR[a]	54	1,267	KF265263
TLR1LB	avTLR1LBF[a]	avTLR1LBR[a]	50	1,058	KF265273
TLR3	avTLR3F[a]	avTLR3R[a]	50	1,267	KF265288
TLR4	avTLR4F[a]	AplTLR4R[b]	52	877	KF265299
TLR5	OleTLR5F[b]	PpoTLR5R[b]	56	990	KF265312
TLR7	avTLR7F[a]	avTLR7R[a]	54	1,341	KF265323
TLR15[e]	PpoTLR15F[b]	FnaTLR15R[b]	54	1,207	KF265333-4
TLR21	PpoTLR21F[b]	PpoTLR21R[b]	61	830	KF265339
Psittaciformes: *Cyanoramphus novaezelandiae*					
TLR1LA	avTLR1LAF[a]	avTLR1LAR[a]	58	1,267	KF265257-8
TLR1LB	PcaTLR1LBF[b]	PcaTLR1LBR[b]	60	971	KF265267-8
TLR2B	avTLR2F[a]	avTLR2R[a]	50	1,285	KF265277-8
TLR3	PcaTLR3F[b]	PcaTLR3R[b]	54	1,080	KF265284
TLR4	MunTLR4F[b]	MunTLR4R[b]	55	943	KF265291-2
TLR5	ShaTLR5F[b]	AalTLR5R[b]	50	1,073	KF265306-7
TLR7	AalTLR7F[b]	AalTLR7R[b]	52	831	KF265318
Psittaciformes: *Strigops habroptilus*					
TLR1LA	avTLR1LAF[a]	avTLR1LAR[a]	58	1,267	KF265264
TLR2B	avTLR2F[a]	avTLR2R[a]	50	1,285	KF265281
TLR3	avTLR3F[a]	avTLR3R[a]	52	1,267	KF265289
TLR4	MunTLR4F[b]	MunTLR4R[b]	55	943	KF265300
TLR5	ShaTLR5F[b]	ShaTLR5R[b]	54	983	KF265313
TLR7	parrotTLR7F[b]	parrotTLR7R[b]	56	677	KF265324
Passeriformes: *Callaeas wilsoni*					
TLR1LA	avTLR1LAF[a]	avTLR1LAR[a]	58	1,267	KF265256
TLR1LB	PcaTLR1LBF[b]	PcaTLR1LBR[b]	56	971	KF265266
TLR3	avTLR3F[a]	avTLR3R[a]	50	1,267	KF265283
TLR5	avTLR5F[a]	avTLR5R[a]	50	1,345	KF265304-5
TLR7[f]	avTLR7F2[a]	PcaTLR7R[b]	50	960	KF265316-7
TLR15	finchTLR15F[b]	avTLR15R[a]	50	1,281	KF265326-7
TLR21	finchTLR21F[c]	finchTLR21R[c]	57	702	KF265335
Passeriformes: *Mohoua ochrocephala*					
TLR1LA	avTLR1LAF[a]	avTLR1LAR[a]	58	1,267	KF265259
TLR1LB	PcaTLR1LBF[b]	PcaTLR1LBR[b]	56	971	KF265269-70
TLR3	PcaTLR3F[b]	PcaTLR3R[b]	55	1,080	KF265285-6
TLR4	PauTLR4F[c]	PauTLR4R[c]	58	722	KF265293-4
TLR5[g]	avTLR5F[a]	avTLR5R[a]	50	1,345	KF265308-9
TLR7[f]	PcaTLR7F[b]	PcaTLR7R[b]	50	891	KF265319
TLR15	avTLR15F[a]	avTLR15R[a]	54	1,337	KF265328-9
TLR21	finchTLR21F[c]	finchTLR21R[c]	57	702	KF265336

Table 2 continued

Species and locus	Forward	Reverse	Ta	Length (bp)	Genbank accession numbers[d]
Passeriformes: *Notiomystis cincta*					
TLR1LA	avTLR1LAF[a]	avTLR1LAR[a]	58	1,267	KF265260
TLR1LB	PcaTLR1LBF[b]	PcaTLR1LBR[b]	56	971	KF265271
TLR2B	avTLR2F[a]	avTLR2R[a]	50	1,285	KF265279
TLR4	avTLR4F[a]	AplTLR4R[b]	52	877	KF265295-6
TLR5	PassTLR5F[b]	AalTLR5R[b]	56	1,136	KF265310
TLR7[f]	avTLR7F2[a]	PcaTLR7R[b]	50	960	KF265320-1
TLR15	finchTLR15F[b]	avTLR15R[a]	52	1,281	KF265330-1
TLR21	finchTLR21F[c]	finchTLR21R[c]	58	702	KF265337-8
Passeriformes: *Philesturnus carunculatus*					
TLR1LA	avTLR1LAF[a]	avTLR1LAR[a]	58	1,267	KF265261-2
TLR1LB	avTLR1LBF[a]	avTLR1LBR[a]	58	1,058	KF265272
TLR2B	avTLR2F[a]	avTLR2R[a]	50	1,285	KF265280
TLR3	avTLR3F[a]	avTLR3R[a]	50	1,267	KF265287
TLR4	PauTLR4F[c]	PauTLR4R[c]	57	722	KF265297-8
TLR5	avTLR5F[a]	avTLR5R[a]	50	1,345	KF265311
TLR7[f]	avTLR7F2[a]	avTLR7R2[a]	57	1,060	KF265322
TLR15	finchTLR15F[b]	avTLR15R[a]	50	1,281	KF265332
Passeriformes: *Xenicus gilviventris*					
TLR1LA	avTLR1LAF[a]	avTLR1LAR[a]	58	1,267	KF265265
TLR1LB	avTLR1LBF[a]	avTLR1LBR[a]	50	1,058	KF265274
TLR3	avTLR3F[a]	PcaTLR3R[b]	50	1,142	KF265290
TLR4	PassTLR4F[b]	PauTLR4R[c]	56	722	KF265301
TLR7[f]	avTLR7F2[a]	PcaTLR7R[b]	50	960	KF265325

[a] Alcaide and Edwards (2011); [b] developed in the current study (Table 1); [c] Grueber et al. (2012)

[d] For species in which $N = 2$ (all except *X. gilviventris* where $N = 1$); only one accession is provided when both individuals carried identical sequence

[e] Thermocycling protocol repeated 40× (not 35× as for all other loci)

[f] Note that *TLR7* may be duplicated in Passerines [see Grueber et al. (2012) and references therein]

[g] Amplifies two clear bands, of which the smaller is the target product

2011; Grueber et al. 2012; Table 2) for 40 s, and extension at 72 °C for 80 s, with a final extension step of 72 °C for 10 min. Products were purified by excision from 1 % agarose, cleaned up using the MEGAquick-spin total fragment DNA purification kit (iNtRON Biotechnology Inc.), and sequenced in both directions on an ABI 3730xl Genetic Analyser (service provided by Genetic Analysis Services at Otago). Sequences were edited using Sequencher v5.0 (Gene Codes Corporation), using IUPAC ambiguity codes where double-peaks were observed. Correct amplification of the target sequence was confirmed using BLAST (Altschul et al. 1997) search.

We developed a total of 30 new primers (Table 1) which, along with cross-species amplification of previously-published primers, enabled us to successfully obtain on average 1,087 bp fragments (range 677–1,345 bp) for between five and eight TLR genes in nine bird species across four families (amplification conditions and Genbank accession numbers provided in Table 2). Although we have not amplified the full sequence of each gene, our primers target their extracellular domains: the variable region responsible for pathogen binding (as opposed to the intracellular domain, which is highly conserved to maintain the integrity of intracellular signalling cascades; Areal et al. 2011). Many of these primers will likely work well in related species. These primers will be used to survey TLR diversity in threatened populations, and for addressing more general questions regarding avian TLR evolution and the role of immunogenetic diversity after population bottlenecks.

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