Selective constraint acting on TLR2 and TLR4 genes of Japanese *Rana* frogs

Quintin Lau [Corresp., 1] , Takeshi Igawa [2] , Tiffany A Kosch [3] , Yoko Satta [1]

[1] Department of Evolutionary Studies of Biosystems, Sokendai (Graduate University for Advanced Studies), Hayama, Kanagawa, Japan

[2] Amphibian Research Center, Hiroshima University, Higashi-Hiroshima, Hiroshima, Japan

[3] One Health Research Group, College of Public Health, Medical and Veterinary Sciences, James Cook University of North Queensland, Townsville, Queensland, Australia

Corresponding Author: Quintin Lau
Email address: quintin@soken.ac.jp

Toll like receptors (TLRs) are an important component of innate immunity, the first line of pathogen defence. One of the major roles of TLRs includes recognition of pathogen-associated molecular patterns. Amphibians are currently facing population declines and even extinction due to chytridiomycosis caused by the *Batrachochytrium dendrobatidis* (Bd) fungus. Evidence from other vertebrates shows that TLR2 and TLR4 are involved in innate immunity against various fungi. Such genes therefore may play a functional role in amphibian-chytridiomycosis dynamics. Frogs from East Asia appear to be tolerant to Bd, so we examined the genetic diversity that underlies TLR2 and TLR4 from three Japanese Ranidae frog species, *Rana japonica*, *R. ornativentris* and *R. tagoi tagoi* (n = 5 per species). We isolated 27 TLR2 and 20 TLR4 alleles and found that these genes are evolutionarily conserved, with overall evidence supporting purifying selection. In contrast, site-by-site analysis of selection identified several specific codon sites under positive selection, some of which were located in the variable leucine rich repeat domains. In addition, preliminary expression levels of TLR2 and TLR4 from transcriptome data showed overall low expression. Although it remains unclear whether infectious pathogens are a selective force acting on TLRs of Japanese frogs, our results support that certain sites in TLRs of these species may have experienced pathogen-mediated selection.

1   **Selective constraint acting on TLR2 and TLR4 genes of Japanese *Rana* frogs.**

2   Quintin Lau[1], Takeshi Igawa[2], Tiffany A. Kosch[3], Yoko Satta[1]

3   [1]Department of Evolutionary Studies of Biosystems, Sokendai (The Graduate University for Advanced

4   Studies), Kamiyamaguchi 1560-35, Hayama, Kanagawa 240-0193, Japan.

5   [2]Amphibian Research Center, Hiroshima University, 1-3-1, Higashi-Hiroshima, Hiroshima 739-8526,

6   Japan.

7   [3]One Health Research Group, College of Public Health, Medical and Veterinary Sciences, James Cook

8   University, Townsville, Queensland, 4811, Australia.

9   Corresponding author: Quintin Lau, email: quintin@soken.ac.jp , Tel: +81 46 858 1610, Fax: +81 46 858

10   1544

11   Takeshi Igawa, email: tigawa@hiroshima-u.ac.jp

12   Tiffany A Kosch, email: tiffany.kosch@jcu.edu.au

13   Yoko Satta, email: satta@soken.ac.jp

14   **Abstract**

15   Toll like receptors (TLRs) are an important component of innate immunity, the first line of pathogen

16   defence. One of the major roles of TLRs includes recognition of pathogen-associated molecular patterns.

17   Amphibians are currently facing population declines and even extinction due to chytridiomycosis caused

18   by the *Batrachochytrium dendrobatidis* (Bd) fungus. Evidence from other vertebrates shows that TLR2

19   and TLR4 are involved in innate immunity against various fungi. Such genes therefore may play a

20   functional role in amphibian-chytridiomycosis dynamics. Frogs from East Asia appear to be tolerant to

21   Bd, so we examined the genetic diversity that underlies TLR2 and TLR4 from three Japanese Ranidae

22   frog species, *Rana japonica*, *R. ornativentris* and *R. tagoi tagoi* (n = 5 per species). We isolated 27 TLR2

23   and 20 TLR4 alleles and found that these genes are evolutionarily conserved, with overall evidence

24   supporting purifying selection. In contrast, site-by-site analysis of selection identified several specific

25   codon sites under positive selection, some of which were located in the variable leucine rich repeat

26   domains. In addition, preliminary expression levels of TLR2 and TLR4 from transcriptome data showed

27   overall low expression. Although it remains unclear whether infectious pathogens are a selective force

28   acting on TLRs of Japanese frogs, our results support that certain sites in TLRs of these species may have

29   experienced pathogen-mediated selection.

30

31 **Introduction**

32 Toll-like receptors (TLRs) are a type of pattern recognition receptor that recognize pathogen-associated

33 molecular patterns (PAMPs) such as bacterial cell walls and nucleic acids (Medzhitov, 2001). The signalling of

34 TLRs triggers the synthesis and release of pro-inflammatory cytokines, and thus TLRs have an important role

35 in innate immunity and activation of adaptive immunity. TLRs are type 1 membrane glycoproteins comprised

36 of extracellular and cytoplasmic domains; the extracellular domain is also considered as a 'pathogen-

37 recognition domain' with a variable number of leucine rich repeats (LRR, protein motifs in the ectodomain

38 inferred to be important for recognising molecules), while the cytoplasmic signalling domain is a conserved

39 toll/IL-1 domain (Mikami et al., 2012). The TLR repertoire differs between vertebrate groups ranging from 10

40 loci in humans and great apes to 21 in urodele amphibians (Takeda, Kaisho & Akira, 2003; Quach et al., 2013;

41 Babik et al., 2014). Specific TLR loci are generally considered to recognize different groups of PAMPs, for

42 example, TLR1 and TLR6 associate with TLR2 to recognize acylated lipopeptides, TLR4 recognize

43 lipolysaccharides in gram-negative bacteria, and TLR5 recognize bacterial flagellin (Poltorak et al., 1998;

44 Voogdt et al., 2016). In addition, TLR2 and TLR4 can recognize PAMPs derived from different fungi species

45 (reviewed by Roeder et al., 2004; Luther & Ebel, 2006).

46 In amphibians, TLRs have been described in *Xenopus* frogs (20 loci from 14 families, Ishii et al., 2007) and

47 newts (21 loci from 13 families, Babik et al., 2014). TLR families characterized in both frogs and newts include

48 TLR01, TLR02, TLR03, TLR05, TLR07, TLR08, TLR09, TLR12, TLR13, TLR14, TLR21, and TLR22. However, the

49 prevalence of TLR4 across amphibian taxa is uncertain: no TLR4 orthologs were found in newts, but putative

50 TLR4 were identified in *Xenopus* (Ishii et al., 2007; Babik et al., 2014). In addition, TLR4 was one of the 11 TLR

51 genes that were isolated from transcriptome data of *Bombina maxima* frogs (Zhao et al., 2014).

52 Most TLR genes are functionally constrained, and typically have high sequence conservation and slow

53 evolutionary rates to maintain a functional role of recognizing conserved PAMPs (Roach et al., 2005),

54 although extracellular LRR domains have higher evolutionary rates compared to intracellular domains

55   (Mikami et al., 2012). An overall signature of purifying selection was identified in TLRs of urodele amphibians,

56   although a few individual codons were found to be evolving under positive selection (Babik et al., 2014), as

57   has been observed in other vertebrates (Wlasiuk & Nachman, 2010; Shang et al., 2018). The identification of

58   TLR-disease associations in vertebrates (Tschirren et al., 2013; Noreen & Arshad, 2015) also supports that

59   episodic selection can occur in TLR genes as a response to changes in pathogen diversity. In addition, human

60   TLR4 displayed significantly negative Tajima's D values in nonsynonymous variants (Smirnova et al., 2001),

61   and subsequent evidence supporting selection for rare TLR4 variants was found (Smirnova et al., 2003).

62   Therefore, while TLRs may be under functional constraints, there is evidence across vertebrates that positive

63   selection may act on these genes in response to local pathogens.

64   Chytridiomycosis is a disease in amphibians caused by the fungal pathogen *Batrachochytridium dendrobatidis*

65   (Bd). This disease has been linked to the decline of amphibian populations worldwide (Daszak, Cunningham &

66   Hyatt, 2003; Longcore et al., 2007; Skerratt et al., 2007; Wake & Vredenburg, 2008). Despite Bd being

67   prevalent in Korea and Japan (Goka et al., 2009; Bataille et al., 2013), within endemic East Asian frogs there is

68   no evidence of Bd-related declines and no published reports of Bd susceptibility following experimental

69   infection; this supports that such frogs could be Bd-tolerant. Additionally, genetic evidence for high Bd

70   genetic diversity and endemism in this region indicates that Bd is endemic to Asia (Fisher, 2009; Bataille et al.,

71   2013), suggesting a long co-evolutionary history between the Bd pathogen and Asian amphibians.

72   While adaptive immune genes of Japanese frogs have been studied in the context of diseases like

73   chytridiomycosis (Lau et al., 2016, 2017), there are limited studies involving innate immunity genes including

74   TLRs. Since TLR2 and TLR4 have been shown to play a role in innate immune responses to various fungi

75   (Roeder et al., 2004; Luther & Ebel, 2006), TLRs therefore may be involved in Bd resistance (Richmond et al.,

76   2009).

77   Basal expression of TLRs can provide an overview of the function of these genes, and have been examined in

78   a few anuran species including *Bombina* and *Xenopus* frogs. In *B. maxima*, expression levels of TLR2 and TLR4,

79   measured using with quantitative RT-PCR, were variable between different adult tissues (Zhao et al., 2014). In

80   *X. laevis*, ubiquitous expression of both TLR2 and TLR4 was detected in adults and tadpoles using standard

81   PCR, but expression levels were not quantified (Ishii et al., 2007). This current study provides the opportunity

82   to expand the knowledge about basal TLR expression in amphibians.

83   To better understand the gene complements of anuran innate immunity, here we characterize the genetic

84   diversity and selection patterns of two candidate TLR genes in three Japanese *Rana* species using molecular

85   cloning and sequence analyses. Given the potential immunological importance of TLRs against fungal

86   pathogens, we hypothesized that TLRs would be subjected to purifying selection in species which show

87   marked resistance to Bd. In addition, we also conducted preliminary investigation of TLR expression using

88   published transcriptomic data, to determine whether basal expression is similar across different life stages

89   and tissue types.

90   **Methods**

91   *Animals*

92   All sample collection was approved by Hiroshima University Animal Research Committee, approval number

93   G14-2. Adult skin tissues were collected from three common Ranidae frog species from Japan (n = 5 per

94   species): the Japanese brown frog (*Rana japonica*), the montane brown frog (*Rana ornativentris*), and Tago's

95   brown frog (*Rana tagoi tagoi*). All frogs are the same individuals used previously to characterize MHC class I

96   and II genes (Lau et al., 2016, 2017, Table S1). All animals were housed in laboratory conditions for a

97   minimum of five weeks and exhibited no clinical signs of disease prior to euthanasia, and thus considered

98   'healthy'. Animals were euthanized through immersion in tricaine methanesulfonate (MS222, 0.5–3 g/L

99   water), and preserved in RNAlater (Applied Biosystems, Carlsbad, CA, USA) at -20ºC prior to excision of skin

100   sample and genomic DNA (gDNA) extraction using DNAsuisui-F (Rizo Inc., Tsukuba, Japan) following

101   manufacturer's protocol.

102 *Isolation of TLR genes from transcriptome data set and primer design*

103 To isolate TLR2 and TLR4 genes, we utilised the published transcriptomic data set previously compiled using

104 Illumina sequences from immune tissues of the three species (Lau et al., 2017). Briefly, we used the

105 assembled transcripts that were annotated with NCBI-BLAST-2.3.30 against the Swissprot protein database

106 (httpr://www.expasy.ch/sprot), and isolated all transcripts that had top hits from BLAST search to published

107 TLR genes of other vertebrates. We manually scanned the NCBI-BLAST search results and aligned each

108 transcript with orthologous genes from *Xenopus laevis* and *Nanorana parkeri* (Xenbase,

109 http://www.xenbase.org/, RRID:SCR_003280, and GenBank accession numbers XM_002933491,

110 XM_018557931, XM_018232906, XM_018565865). Due to low coverage of sequence data, full-length contigs

111 were not available for TLR2 and TLR4 genes in all three species (Figures S1 and S2); in such cases, fragmented

112 contigs were used in the alignment. The genomic structure of most vertebrate TLR genes are unique in that

113 the majority of their coding sequence is located within a single exon. From the alignments, we used Primer 3

114 (Rozen & Skaletsky, 1998) to design degenerate primers that amplified 2348 bp and 2072 bp fragments

115 within a single exon of TLR2 (RanaTLR2_F: 5'-TGRTTGCATACATATGGAGTTG-3', RanaTLR2_R: 5'-

116 GTGGTCCTCTGGCTGAAGAG-3') and TLR4 (RanaTLR4_F: 5'-CTGGCAAGCCTTTCTGAACT-3', RanaTLR4_R: 5'-

117 AGCGGARCATCAACTTTACG-3'), respectively, across all three species (Table S1).

118 *TLR PCR and sequencing*

119 Polymerase chain reaction (PCR) amplification was conducted in Applied Biosystems® Veriti® thermal cycler

120 in 10 μL reactions with 0.25 U TaKaRa Ex Taq® polymerase (Takara Bio Inc.), 1x Ex Taq PCR buffer, 0.2 mM

121 each dNTP, and 0.7 μM each primer and 0.5 – 1.0 μL skin gDNA samples (n = 5 per species) with the following

122 cycle condition: initial Taq activation at 95℃ for 1 min, then 35 cycles of 30-s denaturation at 95℃, 30-s

123 annealing at 60℃ (TLR2) or 61℃ (TLR4), and 80-s extension at 72℃, then a final extension of 72℃ for 3 min.

124 Since TLR alleles could not be phased in heterozygous individuals by sequencing alone, we used molecular

125 cloning following by Sanger sequencing. PCR products were ligated into T-Vector pMD20 (Takara Bio Inc)

126   using DNA Ligation Kit 2.1 (Takara Bio Inc.) and incubated for 30 min at 16°C. For cloning, ligation reactions

127   were transformed into JM109 competent cells (Takara Bio Inc.) and cultured on selective LB plates containing

128   50 μg /mL ampicillin overnight at 37°C. We then amplified positive clones (4 – 10 per individual reaction)

129   using M13 primers and similar PCR conditions, and purified using ExoSAP-IT® (Affymetric Inc., Santa Clara,

130   USA). As amplicons were over 2kbp length, we utilised four to six additional sequencing primers (Table S2,

131   Figures S3 and S4) in addition to M13 primers for sequencing with BigDye® Terminator Cycle Sequencing kit

132   (Applied Biosystems, Foster City, USA) and ABI 3130xl automated sequencer.

133   *Sequence analyses, d_N/d_S comparison with other genes, and selection tests*

134   We measured polymorphism and divergence of the TLR2 and TLR4 sequences using DnaSP 6.10.03 (Rozas et

135   al., 2017), including number of segregating sites (S), number of alleles (N_A), average number of nucleotide

136   differences (k), nucleotide diversity (π), Tajima's D (D) and normalized Fay and Wu's H (Hn). We calculated

137   synonymous (d_S) and nonsynonymous (d_N) divergence and the ratio (d_N/d_S) between the focal species using

138   MEGA7 (Kumar, Stecher & Tamura, 2016). We then compared the d_N/d_S ratio with that of major

139   histocompatibility complex (MHC) class I and II (average among the three species), which are known to be

140   under balancing selection in these species (Lau et al., 2016, 2017). In addition, we compared with d_N/d_S ratio

141   of all orthologous genes collated from the transcriptome data set of Lau et al (2017). This consisted of over

142   3000 orthologous amino acid sequences from each of the three species that were identified using

143   Proteinortho V5.15 (Lechner et al., 2011). We then extracted nucleotide coding sequences of orthologous

144   genes from transcriptome data sets using a custom python script, and used PhyloTreePruner (Kocot et al.,

145   2013) to align the sequences and remove paralogues. Finally, all sequences were compiled together and

146   maximum likelihood estimates of ω (d_N/d_S) were calculated using CODEML in PAML 4.9 (Yang, 2007).

147   To test for selection, we used McDonald-Kreitman (MK) test in DnaSP 6.10.03 to compare species-wide data

148   with outgroup sequences from distantly related Ranidae frogs (*Odorrana amamiensis* and *O. ishikawae*,

149   transcriptome data, source: T. Igawa, GenBank accession numbers MH165314-MH165317). In addition, we

150   tested for sequence-wide neutral ($d_N = d_S$), purifying ($d_N < d_S$) and positive ($d_N > d_S$) selection using codon-

151   based Z tests with 1000 bootstrap replicates in MEGA7. To infer specific codons as positively selected sites

152   (PSSs) with ω (d_N/d_S) > 1, we used omegaMap version 5.0 (Wilson & McVean, 2006) to perform Bayesian

153   inference on independent alignments for each species and gene, following Lau et al. (2016). Neighbour-

154   joining phylogenetic trees from amino acid alignments (p-distance) were constructed independently for TLR2

155   and TLR4 in MEGA7. Protein domain structures of TLR2 and TLR4 were predicted using SMART (Letunic,

156   Doerks & Bork, 2015).

157   *Expression of TLRs*

158   In order to investigate baseline expression of our candidate TLRs, we extracted expression levels from our

159   transcriptome data set (Lau et al., 2017). RSEM v 1.3.0 (Li & Dewey, 2011) was used to extract trimmed mean

160   log expression ratio, or TMM-normalized values which represent estimated relative RNA production levels

161   (Robinson & Oshlack, 2010), for TLR2 and TLR4 transcripts in each of the 12 samples (Table S1). We compared

162   TMM-normalized values within adults (blood, skin and spleen), using false discovery rate (FDR) cut-off of

163   0.001, to determine whether expression was ubiquitous. In addition, we checked if expression in tadpoles

164   was different to adults, using stage 24 and stage 29 tadpoles (in *R. japonica* and *R.ornativentris*) (Gosner,

165   1960). In the cases where full-length contigs were not available from low sequence coverage (*R. ornativentris*

166   TLR2 and TLR4, and *R. t. tagoi* TLR4), we obtained expression values for each fragmented contig.

167   **Results**

168   We characterized a total of 27 TLR2 and 20 TLR4 alleles from the three study species, all of which were

169   distinctive at the amino-acid level. *Rana japonica* had seven TLR2 and five TLR4 alleles, *R. ornativentris* had

170   ten TLR2 and seven TLR4 alleles*,* and *R. tagoi tagoi* had ten TLR2 and eight TLR4 alleles (Table 1, Figures S3

171   and S4, GenBank accession numbers MG999527-MG999573). All alleles clustered phylogenetically into

172   species-specific clades (Figure 1) and had similar domain structure to that of other frogs (Figure S5). Allelic

173   diversity among species was high (Table 1, Table S3), especially TLR2 in *R. ornativentris* and *R. t. tagoi,* where

174   all individuals were heterozygous with two unique alleles.

175   Selection tests over the entire alignment indicated that TLR2 and TLR4 in the three focal species are under

176   purifying selection (Z-value = 2.66 – 3.83, p < 0.01, Table 2) with the exception of TLR4 in *R. t. tagoi* (Z-value =

177   1.26, p = 0.102). In addition, Tajima's D, normalized Fay and Wu's H, and the McDonald-Kreitman test

178   showed no significant support for selection (Table 1). However, omegaMap analyses identified six to nine

179   positively selected sites (PSSs) in either TLR2 or TLR4 of each of the three focal species (Posterior probability

180   > 99%). Of these, two (22.2%) to six (75.0%) PSSs were located in predicted leucine rich repeat domains

181   (Table 2, Figure S3 and S4). There were no PSSs common in all three species studied, but two PSSs of TLR4

182   (sites D56 and S65) were shared across two species (Table 2, Figure S4).

183   Pairwise comparisons of d_N/d_S ratios between *Rana* species were low and ranged from 0.188 to 0.398 (Table

184   3). These ratios were comparable to that of MHC class 1 α3 and MHC class II β2 domains as well as other

185   transcribed genes within the species (mean d_N/d_S of over 3000 genes = 0.380, Figure 2). In contrast, these

186   ratios were lower than that of MHC class I α1 and α2 domains and class II β1 domain.

187   When we checked published transcriptome data for TLR2 and TLR4 expression, preliminary examination of

188   adult tissues indicated no significant differences between tissue types (blood, spleen, skin) at FDR cut-off of

189   0.0001. Nevertheless, overall expression of TLR2 and TLR4 was low and there were a few consistent trends

190   seen across all three species. This included higher expression of TLR2 in adult skin relative to blood, and

191   lower expression of TLR4 in adult skin relative to either blood or spleen (Table 4). Across life stages,

192   expression of TLR2 and TLR4 was seemingly low in tadpoles relative to adult samples (FDR > 0.0001, n.s.,

193   Table 4).

194   **Discussion**

195   The preliminary characterization of TLR2 and TLR4 in the three focal species here provides a platform for

196   future population genetics studies across the species' distributions, uncovering the full TLR diversity with

197   more targeted PCR and sequencing approaches. Overall allelic diversity of TLR2 and TLR4 appeared to be high,

198   whereby only a few alleles were shared between more than one individual (Table S3). Commonly shared

199   alleles were more apparent in *R. japonica*, likely due to a population bottleneck history in the source

200   population of Etajima (Lau et al., 2016). The presence of the TLR4 gene in amphibians was previously unclear,

201   described as 'putative' in *Xenopus* frogs (Ishii et al., 2007) and 'predicted' from genomic data in *Lithobates*

202   *catesbeianus* and *Nanorana parkeri* (GenBank accessions XP_018421367 and PIO23183; Sun et al., 2015;

203   Hammond et al., 2017), and undetected in newts (Babik et al., 2014). The characterization of TLR4 genes in

204   this study supports the existence of this gene family in anurans, whereby TLR4 alleles of the three *Rana*

205   species were similar in phylogeny and domain structure to that of other frogs.

206   From selection tests, we found overall evidence of purifying selection and no support for sequence-wide

207   positive selection. This agrees with data from other vertebrates, including newts, where TLRs are regarded as

208   conserved with their evolution predominated by purifying selection (Roach et al., 2005; Babik et al., 2014).

209   The d_N/d_S ratios in TLR2 and TLR4 of the *Rana* species studied here were remarkably low compared to that of

210   external domains of MHC class I (α1 and α2 domains) and class II (β1 domain), which are considered to be

211   under balancing selection. However, the low d_N/d_S of TLR2 and TLR4 was comparable to d_N/d_S of MHC class 1

212   α3 and MHC class II β2 domains which are intracellular or not involved in peptide recognition, as well as

213   those of over 3000 transcribed genes within the species. These findings further support that TLR2 and TLR4

214   are under functional constraint.

215   Although we found that most of the TLR2 and TLR4 sequences of the Japanese *Rana* frogs were evolutionarily

216   constrained, we identified evidence of adaptive evolution occurring at individual codon sites in our alignment,

217   similar to other vertebrates studied (Wlasiuk & Nachman, 2010; Shang et al., 2018). When comparing with

218   codon sites predicted to be important for binding of non-fungal ligands (Figures S3 and S4), two PSS

219   identified in *R.ornativentris* TLR2 (Q284 and V299) corresponded to sites in human TLR2 predicted to be

220   involved in ligand binding of lipopeptides (N294 and L312, Jin et al., 2007). In addition, one PSS each of *R.*

221   *tagoi tagoi* (T128) and *R. ornativentris* (Q253) corresponded to human TLR4 sites predicted to be involved in

222   secondary (N268) and phosphate (K388) binding, respectively, of bacterial lipopolysaccharides (Park et al.,

223   2009). Positive selection at identical codon sites across different species, as observed in MHC adaptive

224   immune genes (Lau et al., 2016, 2017), could be driven by a single selective force that is pathogen-related.

225   However, in *Rana* TLRs there were no PSSs shared across all three species studied. As we did not examine the

226   patterns of selection in species which are susceptible to Bd, any link is currently speculative; nevertheless, we

227   cannot rule out the possibility of adaptive evolution, potentially driven by pathogens such as Bd, acting on

228   TLR of the study species in recent evolutionary history.

229   Preliminary examination of TLR2 and TLR4 expression levels extracted from transcriptome data showed

230   overall low expression. While the expression data is derived from single individuals that were housed in

231   disease-free environments, it appears that skin of healthy frogs that are not immune-challenged express TLR2

232   more so than TLR4. However, expression of immune-related genes could be modulated following immune or

233   stress challenges, and future studies should monitor immune gene expression following experimental

234   infection with pathogens like Bd. The adult tissue-specific differences in TLR2 and TLR4 expression from *R.*

235   *ornativentris* in this study were distinct from *B. maxima* (Zhao et al., 2014), but sample size should be

236   increased for both species before further inferences can be made. A previous study in *Xenopus* frogs

237   detected ubiquitous expression of both TLR2 and TLR4 in adults and tadpoles using PCR, but expression levels

238   were not quantified (Ishii et al., 2007). Although we found low TLR expression in tadpoles in this study,

239   further conclusions cannot be made due to limited sampling and overall low TLR expression across the

240   samples. Future quantitative studies can investigate expression level changes of TLRs during development

241   from tadpole to adults, as well as that of other innate and adaptive immune genes extracted from the

242   transcriptome data set (Lau et al., 2017).

243 *Conclusion*

244 In this study, we characterized TLR2 and TLR4 genes from three Japanese *Rana* species. We provide strong

245 evidence of purifying selection acting across the TLR2 and TLR4, and evidence of a few specific codon sites

246 under positive selection. Further research is necessary to determine if the positive selection we detected is

247 due to pathogen-driven selection. Since immunity to infectious diseases is usually polygenetic, our study adds

248 to the growing body of literature related to genes that potentially impact resistance to Bd and other

249 pathogens in amphibians.

250 **References**

251 Babik W., Dudek K., Fijarczyk A., Pabijan M., Stuglik M., Szkotak R., Zielinski P. 2014. Constraint and adaptation in
252   newt Toll-like receptor genes. Genome Biology and Evolution 7:81–95.

253 Bataille A., Fong JJ., Cha M., Wogan GOU., Baek HJ., Lee H., Min MS., Waldman B. 2013. Genetic evidence for a high
254   diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium*
255   *dendrobatidis* in wild Asian amphibians. Molecular Ecology 22:4196–4209.

256 Daszak P., Cunningham AA., Hyatt AD. 2003. Infectious disease and amphibian population declines. Diversity and
257   Distributions 9:141–150.

258 Fisher MC. 2009. Endemic and introduced haplotypes of *Batrachochytrium dendrobatidis* in Japanese amphibians:
259   Sink or source? Molecular Ecology 18:4731–4733.

260 Goka K., Yokoyama J., Une Y., Kuroki T., Suzuki K., Nakahara M., Kobayashi A., Inaba S., Mizutani T., Hyatt AD. 2009.
261   Amphibian chytridiomycosis in Japan: Distribution, haplotypes and possible route of entry into Japan.
262   Molecular Ecology 18:4757–4774.

263 Gosner KL. 1960. A simplified table for staging anuran embryos larvae with notes on identification. Herpetologica
264   16:183–190.

265 Hammond SA., Warren RL., Vandervalk BP., Kucuk E., Khan H., Gibb EA., Pandoh P., Kirk H., Zhao Y., Jones M.,
266   Mungall AJ., Coope R., Pleasance S., Moore RA., Holt RA., Round JM., Ohora S., Walle B V., Veldhoen N.,
267   Helbing CC., Birol I. 2017. The North American bullfrog draft genome provides insight into hormonal
268   regulation of long noncoding RNA. Nature Communications 8:1433.

269 Ishii A., Kawasaki M., Matsumoto M., Tochinai S., Seya T. 2007. Phylogenetic and expression analysis of amphibian
270   *Xenopus* Toll-like receptors. Immunogenetics 59:281–293.

271 Jin MS., Kim SE., Heo JY., Lee ME., Kim HM., Paik SG., Lee H., Lee JO. 2007. Crystal structure of the TLR1-TLR2
272   heterodimer induced by binding of a tri-acylated lipopeptide. Cell 130:1071–1082.

273 Kocot KM., Citarella MR., Moroz LL., Halanych KM. 2013. PhyloTreePruner: A phylogenetic tree-based approach for
274   selection of orthologous sequences for phylogenomics. Evolutionary Bioinformatics 2013:429–435.

275 Kumar S., Stecher G., Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger
276   datasets. Molecular Biology and Evolution 33:1870–1874.

277 Lau Q., Igawa T., Komaki S., Satta Y. 2016. Characterisation of major histocompatibility complex class I genes in
278   Japanese Ranidae frogs. Immunogenetics 68:797–806.

279 Lau Q., Igawa T., Minei R., Kosch TA., Satta Y. 2017. Transcriptome analyses of immune tissues from three Japanese
280   frogs (genus *Rana*) reveals their utility in characterizing major histocompatibility complex class II. BMC
281   Genomics 18:994.

282 Lechner M., Findeiß S., Steiner L., Marz M., Stadler PF., Prohaska SJ. 2011. Proteinortho: Detection of (Co-
283   )orthologs in large-scale analysis. BMC Bioinformatics 12:124.

284 Letunic I., Doerks T., Bork P. 2015. SMART: Recent updates, new developments and status in 2015. Nucleic Acids
285   Research 43:D257–D260.

286 Li B., Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference
287   genome. BMC bioinformatics 12:323.

288 Longcore J., Longcore J., Pessier A., Halteman WA. 2007. Chytridiomycosis widespread in anurans of northeastern
289   United States. Journal of Wildlife Management 71:435–444.

290 Luther K., Ebel F. 2006. Toll-like receptors: Recent advances, open questions and implications for aspergillosis
291   control. Medical Mycology 44:219–227.

292 Medzhitov R. 2001. Toll-like receptors and innate immunity. Nature Reviews Immunology 1:135–145.

293 Mikami T., Miyashita H., Takatsuka S., Kuroki Y., Matsushima N. 2012. Molecular evolution of vertebrate Toll-like
294   receptors: Evolutionary rate difference between their leucine-rich repeats and their TIR domains. Gene
295   503:235–243.

296 Noreen M., Arshad M. 2015. Association of TLR1, TLR2, TLR4, TLR6, and TIRAP polymorphisms with disease
297   susceptibility. Immunologic Research 62:234–252.

298 Park BS., Song DH., Kim HM., Choi BS., Lee H., Lee JO. 2009. The structural basis of lipopolysaccharide recognition
299   by the TLR4-MD-2 complex. Nature 458:1191–1195.

300 Poltorak A., He X., Smirnova I., Liu M., Huffel C Van., Du X., Birdwell D., Alejos E., Silva M., Galanos C., Freudenberg
301   M., Ricciardi-castagnoli P., Layton B., Beutler B. 1998. Defective LPS Signaling in C3H / HeJ and C57BL /
302   10ScCr Mice : Mutations in TLR4 Gene. Science. 282:2085–2088.

303 Quach H., Wilson D., Laval G., Patin E., Manry J., Guibert J., Barreiro LB., Nerrienet E., Verschoor E., Gessain A.,
304   Przeworski M., Quintana-Murci L. 2013. Different selective pressures shape the evolution of toll-like
305   receptors in human and African great ape populations. Human Molecular Genetics 22:4829–4840.

306 Richmond JQ., Savage AE., Zamudio KR., Rosenblum EB. 2009. Toward immunogenetic studies of amphibian
307   chytridiomycosis: linking innate and acquired immunity. BioScience 59:311–320.

308 Roach JC., Glusman G., Rowen L., Kaur A., Purcell MK., Smith KD., Hood LE., Aderem A. 2005. The evolution of
309   vertebrate Toll-like receptors. Proceedings of the National Academy of Sciences of the United States of
310   America 102:9577–9582.

311 Robinson MD., Oshlack A. 2010. A scaling normalization method for differential expression analysis of RNA-seq
312   data. Genome Biology 11:R25.

313 Roeder A., Kirschning CJ., Rupec RA., Schaller M., Weindl G., Korting HC. 2004. Toll-like receptors as key mediators
314   in innate antifungal immunity. Medical Mycology 42:485–498.

315 Rozas J., Ferrer-Mata A., Sánchez-DelBarrio JC., Guirao-Rico S., Librado P., Ramos-Onsins SE., Sánchez-Gracia A.

316   2017. DnaSP 6: DNA sequence polymorphism analysis of large data sets. Molecular Biology and Evolution
317   34:3299-3302.

318   Rozen S., Skaletsky HJ. 1998. Primer3. In: Bioinformatics Methods and Protocols Methods in Molecular Biology. 1–
319   41.

320   Shang S., Zhong H., Wu X., Wei Q., Zhang H., Chen J., Chen Y., Tang X., Zhang H. 2018. Genomic evidence of gene
321   duplication and adaptive evolution of Toll like receptors (TLR2 and TLR4) in reptiles. International Journal of
322   Biological Macromolecules 109:698–703.

323   Skerratt LF., Berger L., Speare R., Cashins S., McDonald KR., Phillott AD., Hines HB., Kenyon N. 2007. Spread of
324   chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4:125–134.

325   Smirnova I., Hamblin MT., McBride C., Beutler B., Rienzo A Di. 2001. Excess of rare amino acid polymorphisms in
326   the toll-like receptor 4 in humans. Genetics 158:1657–1664.

327   Smirnova I., Mann N., Dols A., Derkx HH., Hibberd ML., Levin M., Beutler B. 2003. Assay of locus-specific genetic
328   load implicates rare Toll-like receptor 4 mutations in meningococcal susceptibility. Proceedings of the
329   National Academy of Sciences of the United States of America 100:6075–80.

330   Sun Y-B., Xiong Z-J., Xiang X-Y., Liu S-P., Zhou W-W., Tu X-L., Zhong L., Wang L., Wu D-D., Zhang B-L., Zhu C-L., Yang
331   M-M., Chen H-M., Li F., Zhou L., Feng S-H., Huang C., Zhang G-J., Irwin D., Hillis DM., Murphy RW., Yang H-M.,
332   Che J., Wang J., Zhang Y-P. 2015. Whole-genome sequence of the Tibetan frog *Nanorana parkeri* and the
333   comparative evolution of tetrapod genomes. Proceedings of the National Academy of Sciences 112:E1257–
334   E1262.

335   Takeda K., Kaisho T., Akira S. 2003. Toll-Like Receptors. Annual Review of Immunology 21:335–376.

336   Tschirren B., Andersson M., Scherman K., Westerdahl H., Mittl PRE., Raberg L. 2013. Polymorphisms at the innate
337   immune receptor TLR2 are associated with Borrelia infection in a wild rodent population. Proceedings of the
338   Royal Society B: Biological Sciences 280:20130364–20130364.

339   Voogdt CGP., Bouwman LI., Kik MJL., Wagenaar JA., van Putten JPM. 2016. Reptile Toll-like receptor 5 unveils
340   adaptive evolution of bacterial flagellin recognition. Scientific Reports 6:19046.

341   Wake DB., Vredenburg VT. 2008. Are we in the midst of the sixth mass extinction? A view from the world of
342   amphibians. Proceedings of the National Academy of Sciences of the United States of America 105:11466–
343   11473.

344   Wilson DJ., McVean G. 2006. Estimating diversifying selection and functional constraint in the presence of
345   recombination. Genetics 172:1411–1425.

346   Wlasiuk G., Nachman MW. 2010. Adaptation and constraint at toll-like receptors in primates. Molecular Biology
347   and Evolution 27:2172–2186.

348   Yang Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. Molecular Biology and Evolution 24:1586–
349   1591.

350   Zhao F., Yan C., Wang X., Yang Y., Wang G., Lee W., Xiang Y., Zhang Y. 2014. Comprehensive transcriptome profiling
351   and functional analysis of the frog (*Bombina maxima*) immune system. DNA Research 21:1–13.

Polymorphism of TLR2 and TLR4

Polymorphism of TLR2 and TLR4 in *R. japonica*, *R. ornativentris* and *R. tagoi tagoi*.

1   Table 1. Polymorphism of TLR2 and TLR4 in *R. japonica*, *R. ornativentris* and *R. tagoi tagoi*.

Gene	2N	nsites	N_A	S	k	π	D	Hn	MK P-value
TLR2									
R. japonica	10	2312	7	28	10.48	0.0045	-0.474	-0.873	0.629
R. ornativentris	10	2312	10	44	13.89	0.0060	-0.521	-0.242	1.000
R. tagoi tagoi	10	2312	10	39	13.29	0.0058	-0.292	-1.108	0.215
TLR4									
R. japonica	10	2072	5	41	19.20	0.0093	-0.183	-0.144	0.279
R. ornativentris	10	2078	7	35	14.71	0.0071	0.008	-0.510	0.266
R. tagoi tagoi	10	2072	8	25	8.57	0.0041	-0.584	-1.539	0.127

2   2N: number of gene copies studied; nsites: nucleotide length of sequence; NA: number of alleles; S: number of segregating sites; k: average number of nucleotide
3   differences; π: nucleotide diversity; D: Tajima's D value for all sites (no values were significant at p <0.01); Hn: Fay and Wu's normalized H value for all sites (no
4   values significant p <0.01); MK P-value: McDonald and Kreitman Fisher's exact test P-value

Codon-based Z tests for global selection

Codon-based Z tests for global selection (Z statistics), and specific codon sites under positive selection detected by omegaMap. Codon sites in identical positions in more than one species are underlined.

1 Table 2. Codon-based Z tests for global selection (Z statistics), and specific codon sites under positive
2 selection detected by omegaMap. Codon sites in identical positions in more than one species are
3 underlined.

Gene	Neutrality	Purifying	Positive	Positively selected sites (PSS)
TLR2				
R. japonica	-2.72*	2.69*	-2.63 n.s.	12, 23, 95#, 164, 428#, 672
R. ornativentris	-2.78*	2.66*	-2.77 n.s.	11, 53#, 75#, 207, 284, 299, 417, 509, 535
R. tagoi tagoi	-3.81**	3.83**	-3.76 n.s.	105#, 192, 235, 265, 407, 458#, 485#, 486#
TLR4				
R. japonica	-3.16*	3.11*	-3.11 n.s.	12, 46#, 65#, 129, 133, 221, 339, 430#
R. ornativentris	-3.34*	3.34**	-3.32 n.s.	35#, 56#, 77#, 173, 253#, 378#, 481#, 691
R. tagoi tagoi	-1.28 n.s.	1.28 n.s.	-1.27 n.s.	24, 53#, 56#, 65#, 127, 128, 373#, 416#, 489#

4 *p<0.01, **p<0.001, n.s.- p > 0.05; #PSS located in leucine rich region (LRR)

dN and dS of TLR2, TLR4 and MHC genes

Nonsynonymous (d_N) and synonymous (d_S) divergence between the three focal *Rana* species, for TLR2 and TLR4 genes as well as previously characterized MHC class I and II loci from these three species.

1 Table 3. Nonsynonymous (d_N) and synonymous (d_S) divergence between the three focal *Rana* species,
2 for TLR2 and TLR4 genes as well as previously characterized MHC class I and II loci from these three
3 species.

Gene	d_N	d_S	d_N/d_S
TLR2			
Rj- Ro	0.014	0.077	0.188
Rj-Rt	0.015	0.069	0.213
Ro-Rt	0.020	0.065	0.316
all three species			0.291
TLR4			
Rj- Ro	0.016	0.078	0.207
Rj-Rt	0.018	0.062	0.293
Ro-Rt	0.020	0.051	0.398
all three species			0.379
MHC class I α1	0.092	0.056	1.632
MHC class I α2	0.082	0.054	1.525
MHC class I α3	0.035	0.073	0.476
MHC class II β1	0.139	0.146	0.953
MHC class II β2	0.041	0.151	0.269

Expression of TLR2 and TLR4 across different tissues and life stages based on transcriptome data

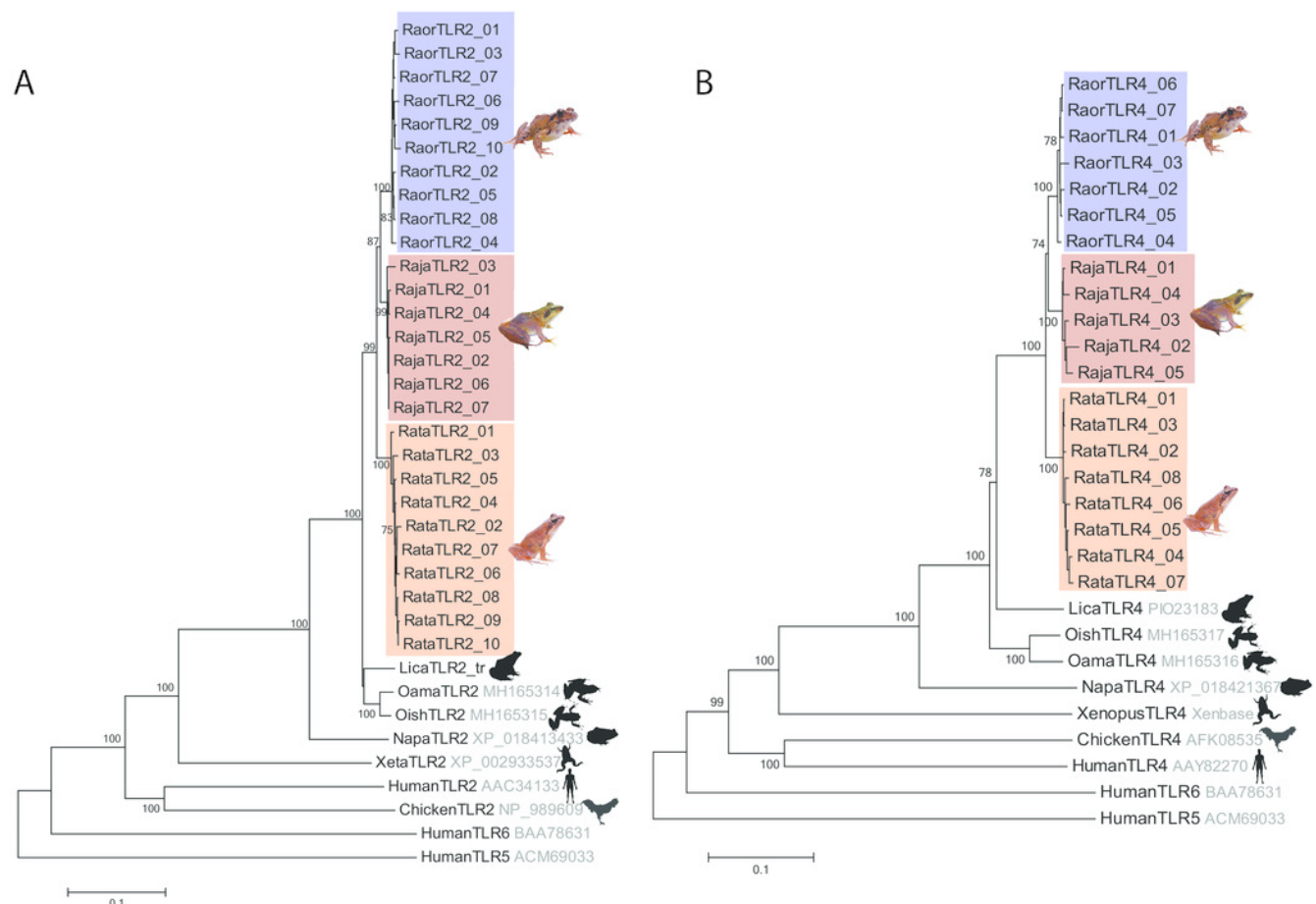
Normalized expression of TLR2 and TLR4 across different tissues and life stages based on transcriptome data set of Lau et al. (2017).

1   Table 4. Normalized expression of TLR2 and TLR4 across different tissues and life stages based on
2   transcriptome data set of Lau et al. (2017).

Gene	Species	Transcript length (bp)	Adult blood	Adult skin	Adult spleen	S24 tadpole	s29 tadpole
TLR2	*R.japonica*	3285	0.25	1.37	2.60	-	0.05
	R. ornativentris						
	fragment 1	1417	0	0.73	0.11	0.20	0
	fragment 2	1324	0.16	0.88	0.22	0.25	0
	R. t. tagoi	2686	0.86	2.55	1.59	-	-
TLR4	*R.japonica*	2830	0.20	0.03	1.14	-	0.01
	R. ornativentris						
	fragment 1	444	0.16	0	0.32	0	0
	fragment 2	548	0	0	0.55	0	0.52
	fragment 3	405	0	0.28	0.58	0	0
	R. t. tagoi						
	fragment 1	1454	0.11	0	0.50	-	-
	fragment 2	629	0.18	0	1.21		

Figure 1

Phylogenetic relationships of TLR2 and TLR4 alleles from three Japanese *Rana* species.

Phylogenetic relationships of (A) TLR2 and (B) TLR4 alleles identified in *R. japonica* (red), *R. ornativentris* (blue) and *R. tagoi tagoi* (orange) and other species based on amino acid alignments (neighbour-joining method). Human TLR5 and TLR6 were used as outgroup sequences. Accession numbers for sequences are indicated. Sequences obtained from transcriptome data include: *Lithobates catesbeianus* (DRA accession number SRP051787), and *Odorrana amamiensis* and *O. ishikawae* (GenBank accessions MH165314-MH165317). Image sources: Q. Lau.

Distribution of d_N/d_S ratios from over 3000 genes isolated from transcriptome data compared to TLR and MHC.

Distribution of d_N/d_S ratios from over 3000 genes isolated from transcriptome data of *R. japonica*, *R. ornativentris* and *R. tagoi tagoi*. d_N/d_S location of TLR2 and TLR4, as well as MHC class I and II domains are marked with arrows.