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

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Toll like receptors (TLRs) are an important component of innate immunity, the first line of pathogen defence, and can recognise pathogen-associated molecular patterns (PAMPs). Amphibians are currently facing population declines and even extinction due to chytridiomycosis caused by the *Batrachochytrium dendrobatidis* (Bd) fungus. TLR2 and TLR4 have been shown in other vertebrates to be involved in innate immunity against various fungi, and such genes may therefore play a functional role in amphibian-chytridiomycosis dynamics. Frogs from East Asia appear to be tolerant to the Bd fungus, so we characterized TLR2 and TLR4 from three Japanese Ranidae frog species, *Rana japonica*, *R. ornativentris* and *R. tagoi tagoi* (n = 5 per species). We found that these genes are evolutionarily conserved, with overall evidence supporting purifying selection. Despite this, we identified specific codon sites that are under positive selection. However, it remains unclear whether the Bd fungus is a selective force acting on TLRs of Japanese frogs.

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13   **Abstract**

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

15   defence, and can recognise pathogen-associated molecular patterns (PAMPs). Amphibians are currently

16   facing population declines and even extinction due to chytridiomycosis caused by the *Batrachochytrium*

17   *dendrobatidis* (Bd) fungus. TLR2 and TLR4 have been shown in other vertebrates to be involved in innate

18   immunity against various fungi, and such genes may therefore play a functional role in amphibian-

19   chytridiomycosis dynamics. Frogs from East Asia appear to be tolerant to the Bd fungus, so we

20   characterized TLR2 and TLR4 from three Japanese Ranidae frog species, *Rana japonica*, *R. ornativentris*

21   and *R. tagoi tagoi* (n = 5 per species). We found that these genes are evolutionarily conserved, with

22   overall evidence supporting purifying selection. Despite this, we identified specific codon sites that are

23   under positive selection. However, it remains unclear whether the Bd fungus is a selective force acting on

24   TLRs of Japanese frogs.

25   **Keywords:** amphibian, toll-like receptors, chytridiomycosis, Ranidae

26 **Introduction**

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

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

29 signalling of TLRs triggers the synthesis and release of pro-inflammatory cytokines, and thus TLRs have an

30 important role in innate immunity and activation of adaptive immunity. TLRs are type 1 membrane

31 glycoproteins comprised of extracellular and cytoplasmic domains; the extracellular domain is also

32 considered as a 'pathogen-recognition domain' with a variable number of leucine rich repeats (LRR),

33 while the cytoplasmic domain is a conserved toll/IL-1 domain (Mikami et al., 2012). The TLR repertoire

34 differs between vertebrate groups ranging from 10 loci in humans and great apes to 21 in urodele

35 amphibians (Takeda, Kaisho & Akira, 2003; Quach et al., 2013; Babik et al., 2014). Specific TLR loci are

36 generally considered to recognize different groups of PAMPs, for example, TLR1 and TLR6 associate with

37 TLR2 to recognize acylated lipopeptides, TLR4 recognize lipolysaccharides in gram-negative bacteria, and

38 TLR5 recognize bacterial flagellin (Poltorak et al., 1998; Voogdt et al., 2016). In addition, TLR2 and TLR4

39 can recognize PAMPs derived from different fungi species (reviewed by Roeder et al., 2004; Luther &

40 Ebel, 2006).

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

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

43 include TLR01, TLR02, TLR03, TLR05, TLR07, TLR08, TLR09, TLR12, TLR13, TLR14, TLR21, and TLR22.

44 However, the prevalence of TLR4 across amphibian taxa is uncertain: no TLR4 orthologs were found in

45 newts, but putative TLR4 were identified in *Xenopus (Ishii et al., 2007; Babik et al., 2014)*. In addition,

46 TLR4, amongst over 11 TLR genes, were isolated from transcriptome data of *Bombina maxima* frogs

47 (Zhao et al., 2014).

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

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

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

51   (Mikami et al., 2012). Overall adaptive constraint was identified in TLRs of urodele amphibians, although

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

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

54   identification of TLR-disease associations in vertebrates (Tschirren et al., 2013; Noreen & Arshad, 2015)

55   also supports that episodic selection can occur in TLR genes as a response to changes in pathogen

56   diversity. In addition, human TLR4 displayed significantly negative Tajima's D values in nonsynonymous

57   variants (Smirnova et al., 2001), and subsequent evidence supporting selection for rare TLR4 variants was

58   found (Smirnova et al., 2003). Therefore, while TLRs may be under functional constraints, there is

59   evidence across vertebrates that positive selection may act on these genes in response to local

60   pathogens.

61   Chytridiomycosis is a fungal disease in amphibians caused by *Batrachochytridium dendrobatidis* (Bd). This

62   disease has been linked to the decline of amphibian populations worldwide (Daszak, Cunningham &

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

64   prevalent in Korea and Japan (Goka et al., 2009; Bataille et al., 2013), there is no evidence of Bd-related

65   declines in East Asian frogs. Additionally, genetic evidence for high Bd genetic diversity and endemism in

66   this region indicates that Bd is endemic to Asia (Fisher, 2009; Bataille et al., 2013), suggesting a long co-

67   evolutionary history between the Bd pathogen and Asian amphibians.

68   While adaptive immune genes of Japanese frogs have been studied in the context of chytridiomycosis

69   (Lau et al., 2016, 2017), there are limited studies involving innate immunity genes including TLRs. Since

70   TLR2 and TLR4 have been shown to play a role in innate immune responses to various fungi (Roeder et

71   al., 2004; Luther & Ebel, 2006), TLRs therefore may be important in Bd resistance (Richmond et al., 2009).

72   Here we characterized two candidate TLR genes in three Bd-tolerant Japanese *Rana* species, to further

73   understand amphibian-chytridiomycosis dynamics.

74   **Methods**

75   *Animals*

76   Adult skin tissues were collected from three common Ranidae frog species from Japan (n = 5 per species):

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

78   brown frog (*Rana tagoi tagoi*). All frogs originated from Hiroshima prefecture in Japan, and are the same

79   individuals used previously to characterize MHC class I and II genes (Lau et al., 2016, 2017, Table S1).

80   Sample collection was approved by Hiroshima University Animal Research Committee, approval number

81   G14-2. All animals were considered 'healthy' as they were housed in laboratory conditions for extended

82   periods and exhibited no clinical signs of disease prior to euthanasia. All sample collection was approved

83   by Hiroshima University Animal Research Committee, approval number G14-2.Animals were euthanized

84   through immersion in tricaine methanesulfonate (MS222, 0.5–3 g/L water), and preserved in RNAlater

85   (Applied Biosystems, Carlsbad, CA, USA) at -20℃ prior to excision of skin sample and genomic DNA

86   (gDNA) extraction using DNAsuisui-F (Rizo Inc., Tsukuba, Japan) following manufacturer's protocol.

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

88   To isolate TLR2 and TLR4 genes, we utilised the transcriptome data set previously compiled using Illumina

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

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

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

92   published TLR genes of other vertebrates. We manually scanned the NCBI-BLAST search results and

93   aligned each transcript with orthologous genes from *Xenopus laevis* and *Nanorana parkeri* (Xenbase,

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

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

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

97 fragmented contigs were used in the alignment. The genomic structure of most vertebrate TLR genes are

98 unique in that they have a majority of their coding sequence located within a single exon. From the

99 alignments, we used Primer 3 (Rozen & Skaletsky, 1998) to design degenerate primers that amplified

100 2348 bp and 2072 bp fragments within a single exon of TLR2 (RanaTLR2_F: 5'-

101 TGRTTGCATACATATGGAGTTG-3', RanaTLR2_R: 5'-GTGGTCCTCTGGCTGAAGAG-3') and TLR4 (RanaTLR4_F:

102 5'-CTGGCAAGCCTTTCTGAACT-3', RanaTLR4_R: 5'-AGCGGARCATCAACTTTACG-3'), respectively, across all

103 three species (Table S1).

104 *TLR PCR and sequencing*

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

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

107 0.2 mM each dNTP, and 0.7 µM each primer and 0.5 – 1.0 µL skin gDNA samples (n = 5 per species) with

108 the following cycle condition: initial Taq activation at 95°C for 1 min, then 35 cycles of 30-s denaturation

109 at 95°C, 30-s annealing at 60°C (TLR2) or 61°C (TLR4), and 80-s extension at 72°C, then a final extension of

110 72°C for 3 min. PCR products were ligated into T-Vector pMD20 (Takara Bio Inc) using DNA Ligation Kit 2.1

111 (Takara Bio Inc.) and incubated for 30 min at 16°C. For cloning, ligation reactions were transformed into

112 JM109 competent cells (Takara Bio Inc.). We then amplified positive clones (4 – 10 per individual

113 reaction) using M13 primers and similar PCR conditions, and purified using ExoSAP-IT® (Affymetric Inc.,

114 Santa Clara, USA). As amplicons were over 2kbp length, we utilised four to six additional sequencing

115 primers (Table S2, Figures S3 and S4) in addition to M13 primers for sequencing with BigDye® Terminator

116 Cycle Sequencing kit (Applied Biosystems, Foster City, USA) and ABI 3130xl automated sequencer.

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

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

119 et al., 2017), including number of segregating sites (S), number of alleles (N_A), nucleotide diversity (π),

120 Tajima's D (D) and normalized Fay and Wu's H (Hn). We calculated synonymous (d_S) and nonsynonymous

121 (d_N) divergence and the ratio (d_N/d_S) between the focal species using MEGA7 (Kumar, Stecher & Tamura,

122 2016). We then compared the d_N/d_S ratio with that of major histocompatibility complex (MHC) class I and

123 II, which are known to be under balancing selection in these species (Lau et al., 2016, 2017). In addition,

124 we compared with d_N/d_S ratio of all orthologous genes collated from the transcriptome data set of Lau et

125 al (2017). This consisted of over 3000 orthologous amino acid sequences from each of the three species

126 that were identified using Proteinortho V5.15 (Lechner et al., 2011). We then extracted nucleotide coding

127 sequences of orthologous genes from transcriptome data sets using a custom python script, and used

128 PhyloTreePruner (Kocot et al., 2013) to align the sequences and remove paralogues. Finally, all sequences

129 were compiled together and maximum likelihood estimates of ω (d_N/d_S) were calculated using CODEML

130 in PAML 4.9 (Yang, 2007).

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

132 data with outgroup sequences from distantly related Ranidae frogs (*Odorrana amamiensis* and *O.*

133 *ishikawae*, transcriptome data, source: T. Igawa). In addition, we tested for sequence-wide neutral (d_N =

134 d_S), purifying ($d_N < d_S$) and positive ($d_N > d_S$) selection using codon-based Z tests with 1000 bootstrap

135 replicates in MEGA7. To infer specific codons as positively selected sites (PSSs) with ω (d_N/d_S) > 1, we used

136 omegaMap version 5.0 (Wilson & McVean, 2006) to perform Bayesian inference on independent

137 alignments for each species and gene, following Lau et al. (2016). Neighbour-joining phylogenetic trees

138 from amino acid alignments (p-distance) were constructed independently for TLR2 and TLR4 in MEGA7.

139 Protein domain structures of TLR2 and TLR4 were predicted using SMART (Letunic, Doerks & Bork, 2015).

140 *Expression of TLRs*

141 We used our transcriptome data set (Lau et al., 2017) to compare expression levels of TLR2 and TLR4

142 between tissue types and adult frogs and tadpoles. RSEM v 1.3.0 (Li & Dewey, 2011) was used to extract

143 trimmed mean log expression ratio, or TMM-normalized values (Robinson & Oshlack, 2010), for TLR2 and

144   TLR4 transcripts in each of the 12 samples (Table S1). In the cases where full-length contigs were not

145   available from low sequence coverage (*R. ornativentris* TLR2 and TLR4, and *R. t. tagoi* TLR4), we obtained

146   expression values for each fragmented contig.

147   **Results**

148   We characterized a total of 27 TLR2 and 20 TLR4 alleles from the three study species distinctive at the

149   amino-acid level: seven TLR2 and five TLR4 alleles in *R. japonica*, ten TLR2 and seven TLR4 alleles *R.*

150   *ornativentris*, and ten TLR2 and eight TLR4 alleles in *R. tagoi tagoi* (Table 1, Figures S3 and S4, GenBank

151   accession numbers MG999527-MG999573). All alleles clustered phylogenetically into species-specific

152   clades (Figure 1) with similar domain structure to that of other frogs (Figure S5). Allelic diversity among

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

154   individuals were heterozygous with two unique alleles. Only a few alleles were shared between more

155   than one individual (Table S3); this was apparent in *R. japonica* and may be due to a population

156   bottleneck history in the source population of Etajima (Lau et al., 2016).

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

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

159   (Z-value = 1.26, p = 0.102). In addition, Tajima's D, normalized Fay and Wu's H and the McDonald-

160   Kreitman test showed no significant support for selection (Table 1). However, omegaMap analyses

161   identified six to nine positively selected sites (PSSs) in either TLR2 or TLR4 of each of the three focal

162   species (Posterior probability > 99%), of which two (22.2%) to six (75.0%) PSSs were located in predicted

163   leucine rich repeat domains (Table 2, Figure S3 and S4). There were no PSSs common in all three species

164   studied, but two PSSs of TLR4 (D56 and S65) were shared across two species (Table 2, Figure S4).

165   The low average number of non-synonymous differences per non-synonymous site (d_N) resulted in low

166   d_N/d_S ratio of between 0.188 to 0.398 in TLR pairwise comparisons between *Rana* species (Table 3). Such

167   ratios were lower than MHC class I α1 and α2 domains and class II β1 domain, but comparable to that of

168   MHC class 1 α3 and MHC class II β2 domains as well as other transcribed genes within the species (mean

169   d_N/d_S of over 3000 genes = 0.380, Figure 2).

170   Initial examination of expression level differences between tissue types within adults (blood, spleen, skin)

171   indicated that overall expression of TLR2 and TLR4 is low but there were a few consistent trends seen

172   across all three species. TLR2 had high expression in adult skin relative to blood, while TLR4 had the

173   lowest expression in adult skin relative to both blood and spleen (Table 4). Expression of TLR2 and TLR4

174   was seemingly low in tadpoles relative to adult samples (Table 4).

175   **Discussion**

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

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

178   more targeted PCR and sequencing approaches. The presence of the TLR4 gene in amphibians was

179   previously unclear, described as 'putative' in *Xenopus* frogs and 'predicted' in *Lithobates catesbeiana* and

180   *Nanorana parkeri* (GenBank accessions XP_018421367 and PIO23183), and undetected in newts. The

181   characterization of TLR4 genes in this study supports the existence of this gene family in anurans,

182   whereby TLR4 alleles of the three *Rana* species were similar in phylogeny and domain structure with that

183   of other frogs.

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

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

186   regarded as conserved with their evolution predominated by purifying selection (Roach et al., 2005;

187   Babik et al., 2014). The low d_N/d_S ratios in TLR2 and TLR4 of the *Rana* species studied here were

188   remarkably low compared to that of external domains of MHC class I (α1 and α2 domains) and class II (β1

189   domain), which are considered to be under balancing selection. However, the low d_N/d_S of TLR2 and TLR4

190   was comparable to d_N/d_S of MHC class 1 α3 and MHC class II β2 domains which are intracellular or not

191   involved in peptide recognition, as well as that of over 3000 transcribed genes within the species. These

192   findings further support that TLR2 and TLR4 are under functional constraint.

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

194   evolutionarily constrained, we identified evidence of adaptive evolution occurring at individual codon

195   sites in our alignment, similar to other vertebrates studied (Wlasiuk & Nachman, 2010; Shang et al.,

196   2018). When comparing with codon sites predicted to be important for binding of non-fungal ligands

197   (Figures S3 and S4), two PSS identified in *R.ornativentris* TLR2 (Q284 and V299) corresponded to sites in

198   human TLR2 predicted to be involved in ligand binding of lipopeptides (N294 and L312, Jin et al., 2007).

199   In addition, one PSS each of *R. tagoi tagoi* (T128) and *R. ornativentris* (Q253) corresponded to human

200   TLR4 sites predicted to be involved in secondary (N268) and phosphate (K388) binding, respectively, of

201   bacterial lipopolysaccharides (Park et al., 2009). Positive selection at identical codon sites across different

202   species, as observed in MHC adaptive immune genes (Lau et al., 2016, 2017), could be driven by a single

203   selective force that is pathogen-related. However, in *Rana* TLRs there were no PSSs shared across all

204   three species studied. Nevertheless, we cannot rule out the possibility of adaptive evolution, potentially

205   driven by pathogens such as the Bd fungus, acting on TLR of the study species in recent evolutionary

206   history.

207   Preliminary examination of TLR2 and TLR4 expression levels extrapolated from transcriptome data

208   showed overall low expression. While the expression data is derived from single individuals that were

209   housed in disease-free environments, it appears that skin of healthy frogs that are not immune-

210   challenged express TLR2 more so than TLR4. However, expression of immune-related genes could be

211   modulated following immune or stress challenges, and future studies should monitor immune gene

212   expression following experimental infection with pathogens like Bd. A previous study in *Xenopus* frogs

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

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

215   study, further conclusions cannot be made due to limited sampling and overall low TLR expression across

216   the samples. Future quantitative studies can investigate expression level changes of TLRs during

217   development from tadpole to adults, as well as that of other innate and adaptive immune genes

218   extrapolated from the transcriptome data set (Lau et al., 2017).

219   *Conclusion*

220   In this study, we characterized TLR2 and TLR4 genes from three Japanese *Rana* species, considered to be

221   tolerant to the deadly Bd fungus. We provide strong evidence of purifying selection acting across the

222   TLR2 and TLR4, and evidence of a few specific codon sites under positive selection. Further research is

223   necessary to determine if the positive selection we detected is due to pathogen-driven selection from the

224   Bd pathogen. Since immunity to pathogens like the Bd fungus is usually polygenetic, our study adds to

225   the growing body of literature related to genes that impact Bd resistance and further contributes to

226   understanding complex amphibian-chytridiomycosis dynamics.

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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 significant p <0.01); Hn: Fay and Wu's normalized H value for all sites (no values
4 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

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.

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

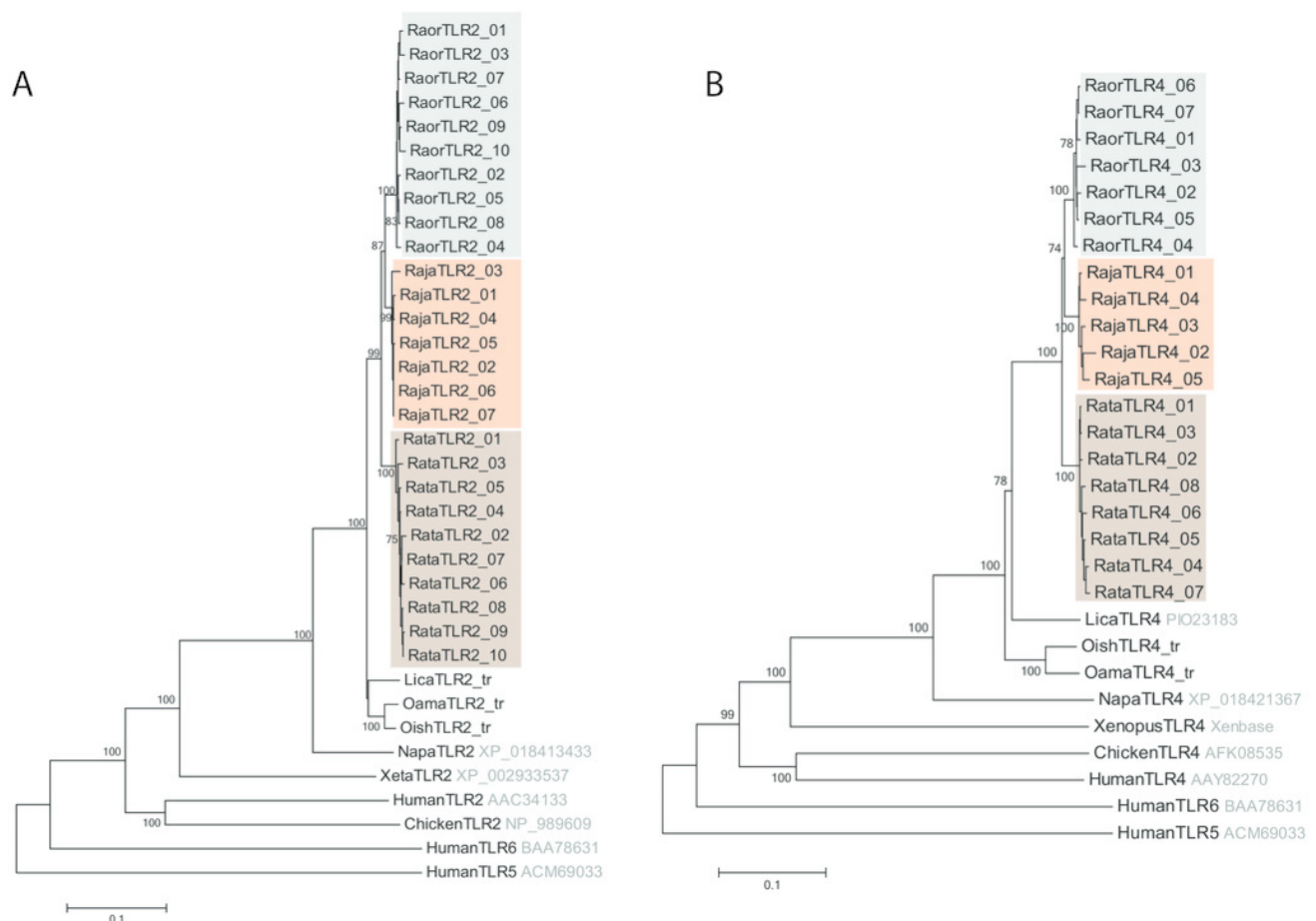
Normalized expression of TLR2 and TLR4 across different tissues and life stages based on transcriptome data set of Lau et al. (2017).

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).

	Transcript length (bp)	Adult blood	Adult skin	Adult spleen	S24 tadpole	s29 tadpole
Raja_TLR2	3285	0.25	1.37	2.60	-	0.05
Raja_TLR4	2830	0.20	0.03	1.14	-	0.01
Raor_TLR2						
fragment 1	1417	0	0.73	0.11	0.20	0
fragment 2	1324	0.16	0.88	0.22	0.25	0
Raor_TLR4						
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
Rata_TLR2	2686	0.86	2.55	1.59	-	-
Rata_TLR4						
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* (orange), *R. ornativentris* (grey) and *R. tagoi tagoi* (brown) 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 catesbeiana* (DRA accession number SRP051787), and *Odorrana amamiensis* and *O. ishikawae* (source: T. Igawa).

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.