- Conservation Genetics -

Transcriptome annotation reveals minimal immunogenetic diversity among Wyoming toads, *Anaxyrus baxteri*

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Abstract

Briefly considered extinct in the wild, the future of the Wyoming toad (*Anaxyrus baxteri*) continues to rely on captive breeding to supplement the wild population. Given its small natural geographic range and history of rapid population decline at least partly due to fungal disease, investigation of the diversity of key receptor families involved in the host immune response represents an important conservation need. Population decline may have reduced immunogenetic diversity sufficiently to increase the vulnerability of the species to infectious diseases. Here we use comparative transcriptomics to examine the diversity of toll-like receptors and major histocompatibility complex (MHC) sequences across three individual Wyoming toads. We find reduced diversity at MHC genes compared to bufonid species with a similar history of bottleneck events. Our data provide a foundation for future studies that seek to evaluate the genetic diversity of Wyoming toads, identify biomarkers for infectious disease outcomes, and guide breeding strategies to increase genomic variability and wild release successes.

Keywords: Amphibian, Bufonidae, Immunogenetics, MHC, TLR, Transcriptomics

Introduction

Over the next century, shifts in environmental conditions are predicted to alter the global distribution of wildlife and their pathogens (Hof et al. 2011; Rohr et al. 2013; Palmer 2018; St Leger 2021; Haver et al. 2022). When coupled with already dramatic declines in biodiversity worldwide (Berger et al. 1998; Daszak et al. 2003; Lips et al. 2006; Pounds et al. 2006; Allendorf et al. 2010; Conde et al. 2019), this forecast makes understanding the diversity of core genetic loci involved in host-pathogen interactions an urgent research frontier vital to the conservation of critically endangered species. Assessing the immunogenetic diversity in species that are recovering from recent severe genetic bottlenecks represents an opportunity to assess the degree to which diversity in these highly variable genomic regions is maintained. For many taxonomic classes experiencing historically unprecedented population declines such as Amphibia, immunogenetic assessments remain a relatively unexplored area of research. This lack of immunogenetic studies represents a knowledge gap that is critical for determining how a species is equipped to handle future pathogens and should be included in conservation genetic assessments, especially for species at high risk for future disease.

The Wyoming toad (*Anaxyrus baxteri,* formerly *Bufo baxteri*), a species endemic only to the Laramie Basin in Albany County, Wyoming, United States, represents an exemplary model for such immunogenetic surveys. This species maintained a stable population up until the 1970s when it experienced a rapid decline, was declared endangered in 1984, and thought to be extinct by 1985 (Vincent et al. 2015). While the cause for the rapid decline of the species is not definitively known, dramatic changes in land use practices and the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd) likely played a role (Dickerson et al. 2003; Dreitz 2006; Burton et al. 2009; Hornyak 2012; Polasik et al. 2016). Two years following the presumed extinction of the species, a small population was discovered at Mortenson Lake in Albany County. In 1989 some surviving wild toads were brought into captivity to found breeding

87 colonies and considerable advances have been made in both breeding management and
88 approaches to reintroduction into the wild (Jennings et al. 2001). Rearing toads for release,
89 rather than tadpoles, has reduced release mortality and shortened the time to reach breeding
90 size (Jennings and Anderson 1997) resulting in an *ex situ* population of around 500 individuals
91 (Vincent et al. 2015). Expansion to multiple, privately owned release sites under Safe Harbor
92 Agreements offers further hope for increasing the wild population (Hornyak 2012).

93 Diversity at immune loci is recognized as a critical axis of defense against infections
94 from pathogens (Sommer 2005), including limiting the immunosuppressive effects of Bd (Ellison
95 et al. 2014; Lips 2016). However, assessments of the genetic diversity of the immune system
96 have yet to be widely adopted in most breeding programs (Woodhams et al. 2011). Several
97 studies have indicated a link between the deeply conserved toll-like receptors (TLRs), a family
98 of innate immune receptors that bind well conserved pathogen associated molecular patterns
99 (PAMPs) from a wide array of pathogens, and immunity against important amphibian pathogens
100 (Richmond et al. 2009; Chen and Robert 2011; Varga et al. 2018). TLRs are one of the first
101 defenses in pathogen recognition and immune response that, upon recognition of a PAMP,
102 trigger signal cascades that initiate an immune response (Khan et al. 2019). TLRs bind PAMPs
103 via an extracellular or endosomal ectodomain, comprised of leucine-rich repeat domains
104 (LRRs), and transduce an activation signal via a cytoplasmic toll-IL-1 receptor (TIR) domain.
105 Most research on anuran TLRs has focused on *Xenopus,* with nearly 20 TLRs identified in both
106 *X. laevis* and *X. tropicalis*. As very little is known about anuran TLRs outside of *Xenopus*,
107 quantifying the TLRs present in Wyoming toads and identifying any genetic variation in them will
108 not only be of utility for immediate conservation and breeding efforts, but also improve the
109 understanding of Anuran innate immunity.

110 An additional set of genes essential for immunity are those of the major
111 histocompatibility complex (MHC). The primary role of cell surface-bound MHC class I and class
112 II proteins is to present cytosolic (class I) and extracellular (class II) pathogen associated

113 antigens to immune cells which can then initiate an immune response. Although genetic
114 differences in MHC have been associated with chytrid susceptibility and resistance across
115 anurans (Richmond et al. 2009; Savage and Zamudio 2011; Bataille et al. 2015) the diversity of
116 the MHC within Wyoming toads is not known. These molecules are present on nucleated cells
117 throughout the vertebrate body (class I) or on professional antigen presenting cells (class II).
118 MHC class I complexes include a class I protein that heterodimerizes with β-2-microglobulin
119 (B2M). The role of B2M is primarily structural. Class I proteins possess three extracellular
120 domains (α1, α2 and α3). The α1 and α2 domains bind peptide antigens for displaying to
121 immune cells and these domains exhibit the most sequence diversity. The α3 domain promotes
122 interactions with B2M and tends to be more conserved. MHC class II protein complexes consist
123 of heterodimers of an α chain and a β chain, each of which possess two extracellular domains
124 (α1 and α2 for the class IIα chain and β1 and β2 for the class IIβ chain). Similar to class I, the
125 α1 and β1 domains of class II complexes bind and display peptide antigens and are typically the
126 most diverse in sequence. Correspondingly, the α2 and β2 domains promote dimerization and
127 tend to be more conserved. Given the critical role of MHC in the immune response and its
128 identified importance in vertebrate captive breeding programs (Fulton et al. 2017; Grogan et al.
129 2017; Schenekar and Weiss 2017; Russell et al. 2018; Smallbone et al. 2021), defining the
130 diversity of these receptors in Wyoming toads would provide an invaluable resource for the
131 optimization of strategies to mitigate the impact of disease in recovery plans.

132 Here we deploy RNA-seq based analyses to describe the diversity of TLR and MHC
133 transcripts in Wyoming toads. We first assembled *de novo* transcriptomes from three retired
134 breeders and curated transcripts encoding TLR and MHC proteins. We then assessed the
135 immunogenetic variability between the three individual toads. Finally, we compared our
136 Wyoming toad results to two *Xenopus* species and two closely related bufonid species; the cane
137 toad (*Rhinella marina*) that has undergone severe recent bottleneck events as a result of
138 introductions to Australia and the common toad (*Bufo bufo*) that has not (Lillie et al. 2014; Thörn

et al. 2021). We also compare the Wyoming toad genome at a chromosomal level to other anurans through karyotyping to identify potential chromosomal or genome duplications. Our analyses of these components of the immune system provide the first insights into how variability of immune receptors that are typically characterized as "conserved" or "highly polymorphic" is reflected within species that have experienced population declines in the last century.

Materials and Methods

Sampling protocol

All experiments involving live animals were performed in accordance with relevant institutional and national guidelines and regulations and were approved by the North Carolina State University Institutional Animal Care and Use Committee (protocol 12-127-O). Individual Wyoming toads 6691, 7039 and 7092 were retired male breeders from US Fish and Wildlife Service managed breeding facilities at the Saratoga National Fish Hatchery and Red Buttes Biological Laboratory in Wyoming. Historically, toads were kept in separate subpopulations (A, B & M) in an attempt to increase genetic diversity (Vincent et al. 2015). Group A toads were descended directly from the wild individuals brought into captivity in the late 1980s while group B toads are offspring collected from Mortenson Lake known to be derived from group A. Group M toads are derived from A and B parents. The toads in our study are derived from two of the groups from two breeding facilities. Toads 6691 and 7039 were group A toads from the Saratoga National Fish Hatchery and Red Buttes Biological Laboratory, respectively. Toad 7092 was an M toad from the Saratoga National Fish Hatchery.

Toads were evaluated for chytridiomycosis by quantitative PCR (qPCR). Samples were collected by rolling a dry polyester-tipped swab across the ventral surface of both hindlimbs ten times each and then rolled along the plantar surface of each hindfoot and between the

165 metatarsals five times. A second sample was collected by scraping a scalpel blade 3-4 times
166 across the ventral surface of both hindlimbs and collecting the surface epithelium onto a dry
167 swab. Tips of swabs were placed into cryovials, air dried and stored at -20 °C. Samples were
168 subjected to qPCR as described (Boyle et al. 2004). Toads were euthanized with buffered
169 tricaine methane sulfonate (10 g/L) and a suite of tissues including lungs, liver, kidney, and
170 small intestine were dissected and stored in TRIzol Reagent (Life Technologies) at -80 °C for
171 RNA extraction. Kidney and liver samples were also harvested for primary cell culture.
172
173 *Chromosomal spreads*
174 Kidney and/or liver tissues were finely chopped and added to a 15 mL centrifuge tube
175 containing 2 mL of Hanks Balanced Salt Solution supplemented with 2 mg/mL Collagenase-B, 3
176 mM $CaCl_2$, and 0.1 mg/mL Primocin (Invivogen). The tube was rocked at 32 °C for 14 h and the
177 resulting cell suspension used to seed a T25 tissue culture flask (with 2 µm filtered cap)
178 containing 10 mL of RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM Glutamax
179 (Thermo Fisher), and 0.1 mg/mL Primocin. The primary culture was incubated at 32 °C, 5% CO_2
180 and observed daily until 80% confluent, at which time dividing cells were arrested at metaphase
181 by exposure to Karyomax (50 ng/mL) for 4 h and then harvested using routine hypotonic
182 exposure and methanol/glacial acetic fixation. Fixed cells were dropped onto clean glass slides,
183 air-dried, dehydrated, and stained with 80 ng/mL DAPI. Chromosome images were acquired
184 using an Olympus BX61 microscope equipped with Smart Capture 3, (Digital Scientific,
185 Cambridge, UK) as described previously (Breen et al. 2004).
186
187 *Transcriptome Sequencing, Assembly, & Annotation*
188 RNA was extracted from Wyoming toad lungs, liver, kidney, and small intestine using
189 Qiagen RNeasy Kit. RNA quantity and integrity was assessed with an Agilent Bioanalyzer. For
190 each toad, 0.25 µg of high quality RNA (RNA Integrity numbers >7) from each tissue were

191 pooled for sequencing. RNA was prepared for multiplex sequencing with the TruSeq RNA kit
192 (Illumina) and sequenced (2 x 100 bp PE reads) on two lanes of HiSeq2000 (Illumina). Raw
193 data was deposited into NCBI's Sequence Read Archive (SRA) under study number
194 SRP156163 and accession numbers SRX4501368 (toad 6691), SRX4501367 (toad 7039), and
195 SRX4501369 (toad 7092). Raw reads were trimmed to remove adaptors and poor quality
196 sequences using Trimmomatic (Bolger et al. 2014) and assembled into transcripts using Trinity
197 *de novo* assembler version 02-25-2013 (Grabherr et al. 2011). Transcripts containing bacterial,
198 viral, plasmid or adaptor sequences identified by NCBI's vector trim tool were removed. The
199 resulting assembled transcriptomes were deposited in NCBI's Transcriptome Shotgun Assembly
200 (TSA) database under accession numbers GGUS00000000 (toad 6691), GGUR00000000 (toad
201 7039), and GGUQ00000000 (toad 7092), and BUSCO v3.0.2 was used to identify
202 ultraconserved, single copy genes within the transcriptomes (Simão et al. 2015) employing the
203 tetrapoda ortholog database, odb9 (Zdobnov et al. 2017). Orthologs identified here are
204 expected to be found in all species belonging to a selected order. To assess transcriptome
205 quality, Bowtie2 v2.3.4.1 (Langmead and Salzberg 2012) was used to estimate the percent of
206 raw reads able to be mapped back onto each respective *de novo* transcriptome.
207 The identity of individual transcripts from *de novo* assembled transcriptomes were
208 predicted using the Trinnotate pipeline (Bryant et al. 2017). Transcriptomes were assigned
209 annotations with Kegg, Eggnog, Uniprot, and Uniref90. BlastX and BlastP were performed for
210 the Uniprot and Uniref90 portions of the pipeline to assign gene ontology (GO) terms. An e-
211 value threshold of 10 e-6 was used for each of the annotation stages. To assess the breakdown
212 of transcripts into their biological processes, GO terms and their relative proportions were
213 charted using Bioconductor package GSEABase (Morgan et al. 2018) and custom scripts.
214 Visualizations of GO terms and variants between each individual toad were generated using the
215 ggplot2 package in R studio (Wilkinson 2011). The annotated transcriptome files are available
216 via Dryad (https://doi.org/10.5061/dryad.n2z34tmz9).

217

218 *Manual Identification of Immune Genes*

219 TLR and MHC sequences were identified from the three Wyoming toad *de novo*
220 assembled transcriptomes using an internal custom BLAST server (Priyam et al. 2019). For
221 both TLR and MHC, *X. tropicalis* protein sequences from Xenbase (Fortriede et al. 2020) and
222 NCBI were used as queries (see figures for accession numbers). For TLR genes, well-
223 conserved TIR domains were used as blast queries while full sequences were used as queries
224 for MHC genes. Putative TLR and MHC transcripts were imported into Geneious Prime 2021.2
225 (https://www.geneious.com) where they were translated into amino acid sequences. Protein
226 sequences were assigned as TLR (1-21) or MHC (class I or II) using a combination of BLAST
227 (Altschul et al. 1990), SMART (Letunic et al. 2021), Pfam (Punta et al. 2012), and phylogenetic
228 analysis. *X. laevis*, *X. tropicalis*, common toad and cane toad sequences were acquired from
229 NCBI and Xenbase to use as comparison groups in both TLR and MHC analyses (see figures
230 for accession numbers). For common and cane toads, both predicted genes, as well as
231 sequences present in the reference genome that were identified via BLAST searches, were
232 used in our analyses.

233 Alignments were generated using the Clustal Omega (Sievers and Higgins 2014) plugin
234 in Geneious Prime with no adjustments. We generated alignments for both full length
235 sequences (MHC) and domain only sequences (TLR TIR domains; MHC α or β domains).
236 Domains were determined through a combination of SMART and Pfam analysis as well as
237 manual annotation based on previously published domain assignments (Lillie et al. 2014, 2016;
238 Dirscherl et al. 2014). The evolutionary relationships among genes were estimated using
239 maximum likelihood based phylogenetic analyses in IQ-TREE version 1.6.10 (Minh et al. 2020).
240 Support for inferred relationships was evaluated using 1000 bootstrap replicates. Xenbase
241 nomenclature for TLRs was used to assign names to the putative Wyoming toad TLR

242 sequences. Predicted peptide binding regions for MHC alignments are based on those used by
243 Lillie et al. (2014, 2016).
244
245 *Annotation of Major Histocompatibility Complex (MHC) sequences*
246 As MHC class I and class II have been described in the closely related cane toads (Lillie
247 et al. 2014, 2016), we used these sequences for identifying and cataloging Wyoming toad MHC
248 sequences. We employed the MHC nomenclature guidelines (Klein et al. 1990) for the initial
249 classification of Wyoming toad sequences. For example, class I sequences were named
250 *Anabax*-UX where the six letter abbreviation, *Anabax*, refers to the first three letters of the
251 genus and species, *Anaxyrus baxteri*. The letter "U" (named for "Uno") corresponds to the
252 classical MHC class I lineage, and the variable second letter "X" designates the locus (named
253 alphabetically). Similarly, class II sequences were named *Anabax*-DXA or *Anabax*-DXB, with
254 the first letter D (named for "duo") corresponding to the classical class II lineage, the variable
255 second letter "X" designates the locus (named alphabetically), and the third letter reflecting an α
256 chain (A) or β chain (B). Predicted alleles of the same sequence are indicated with a numeric
257 suffix (e.g. *01, *02).
258
259 **Results**
260
261 *Chromosomal Spreads*
262 We find that Wyoming toads possess eleven pairs of chromosomes (**Fig. 1**), as
263 determined by karyotype analyses using DAPI stained metaphase preparations. This is
264 consistent with at least 20 other diploid bufonid species with which Wyoming toads share recent
265 common ancestry including the American toad (*Bufo americanus*), common toad (*Bufo bufo*),
266 and green toad (*Bufo viridis*) (Gregory 2021). These results suggest Wyoming toads have a
267 similar genome size to most bufonids, demonstrating no evidence for cryptic polyploidy.

268

269 *Transcriptome assembly and annotation*

270 The final *de novo* transcriptomes consisted of 331,616, 199,011 and 209,390 transcripts,
271 for toads 6691, 7039, and 7092, respectively. More than 90% of cleaned reads were accurately
272 mapped onto the respective transcriptome assembly, suggesting high quality transcriptome
273 assemblies. (**Supplementary Table 1**). More than 75% of tetrapod orthologs could be assigned
274 to Wyoming toad transcripts by BUSCO analysis (**Supplementary Fig. S1**) (Manni et al. 2021),
275 suggesting these transcriptomes from selected tissues represent a sufficient survey of the full
276 Wyoming toad transcriptome. Additionally, 15 genes were found to be duplicated in all three
277 toads (**Supplementary Table 2**), though further Gene Ontology enrichment analyses are not
278 possible on so few genes. Analysis of automated transcriptome annotation revealed little
279 variation between the three individuals in terms of sequences annotated. (**Supplementary Fig.
280 S2**).

281

282 *Toll Like Receptor Sequences*

283 Vertebrate TLRs can be classified into six major families: TLR1, TLR3, TLR4, TLR5,
284 TLR7 and TLR11 with some families including multiple TLRs (e.g. TLR7, TLR8 and TLR9 are all
285 within the TLR7 family) (Roach et al. 2005; Ishii et al. 2007; Nie et al. 2018). Although TLR
286 sequence information is publicly available for *Xenopus* and other amphibian species, automated
287 gene annotations have not been verified for Anuran taxa outside of *X. tropicalis*. We find all
288 bufonids surveyed to express members of all six TLR families (**Fig. 2 and Supplementary
289 Table S3**). In the TLR1 family, Wyoming toad, common toad, and cane toad were found to have
290 TLR1, TLR2, and TLR14 sequences. Wyoming toad and cane toad expressed two TLR1
291 sequences: TLR1a and TLR1b. This parallels the multiple TLR1 sequences found in *X.
292 tropicalis* (Roach et al. 2005; Ishii et al. 2007; Nie et al. 2018). In our Wyoming toad
293 transcriptomes, full-length TLR1a and TLR1b were identified in all three individuals while

294 complete TLR2 and TLR14 sequences were only detected in individuals 6691 and 7092.
295 Although truncated transcripts of TLR2 and TLR14 were identified from toad 7039 with identical
296 ectodomains to toads 6691 and 7092, these transcripts were missing a majority of their TIR
297 domain and thus were not included in the phylogeny (**Fig. 2**).

298 The single gene TLR3, TLR4 and TLR5 families are known from *Xenopus* species, and
299 were also found in all bufonid species surveyed, with no variation between the three individual
300 Wyoming toad sequences. Within the TLR7 family, *Xenopu*s encodes TLR7, TLR8, and TL9
301 (Roach et al. 2005; Ishii et al. 2007; Nie et al. 2018). TLR7 was not identified in any bufonid
302 examined. In contrast, two TLR8 sequences (TLR8a and TLR8b) were found across all bufonids
303 examined. However, each of the Wyoming toads expressed only one of the TLR8 sequences.
304 TLR8a was found in toad 7039 and TLR8b was found in toad 6691 and toad 7092. It is currently
305 unclear if TLR8a and TLR8b represent different genes or, perhaps, reflect different haplotypes
306 of the same gene. The common toad also encodes TLR9, but orthologs were not identified in
307 the Wyoming toad or cane toad.

308 Within the TLR11 family, *Xenopus* encodes TLR12, TLR13, TLR21 and TLR22 (Roach
309 et al. 2005; Ishii et al. 2007; Nie et al. 2018). TLR12, TLR13 and TLR21 were identified in all
310 bufonids examined. All Wyoming toad sequences were identical at TIR domains across all
311 individual genes within the TLR11 family, with the exception of TLR12 where individual 6691
312 and 7039 were identical, but differed from 7092 at one amino acid residue. Although *Xenopus*
313 are known to encode a TLR22 sequence, this was not found in any bufonid species surveyed.
314 For all identified sequences in the TLR11 family, there is evidence of only one gene copy per
315 species (**Fig. 2**).

316

317 *MHC class I sequences*

318 Wyoming toads were found to express identical B2M sequences across all individual
319 toads (**Supplemental Table S4**) and four different groups of MHC class I sequences (*Anabax-*

320 *UA*, *Anabax-UB*, *Anabax-UC*, *Anabax-UD*) that likely indicate the presence of at least two and
321 up to four MHC class I gene loci in this species. Two to three transcripts were identified from
322 each group that may reflect haplotypic variation and/or alternative mRNA splicing. Phylogenetic
323 analyses of the Wyoming toad MHC class I α1 - α3 domains with those from cane toad,
324 common toad, *X. laevis* and *X. tropicalis* demonstrate that the Wyoming toad *Anabax-UA*
325 sequences are the most similar to the cane toad MHC class I UA sequence, *Rhimar-UA* (Lillie et
326 al. 2014), whereas *Anabax-UB*, *Anabax-UC*, and *Anabax-UD* sequences are more similar to
327 common toad "F10-like" sequences (**Fig. 3**). Transcripts of *Anabax-UA* are the only ones
328 encoding a prototypical MHC class I structure with a transmembrane domain and we identified
329 two full-length *Anabax-UA* sequences: *Anabax-UA*01* from toad 7039, and *Anabax-UA*02* from
330 toads 6691 and 7092. These have identical signal peptide, α1, α3, and transmembrane domains
331 and cytoplasmic tails, and differ only by four residues in the α2 domain (**Fig 4, Supplemental**
332 **Table S4, and Supplemental Fig S3**).

333 In contrast to *Anabax-UA*, all other class I sequences either encode a stop codon after
334 the α2 (*Anabax-UC*) or are 3' truncated (*Anabax-UB* and *Anabax-UD)*. We identified two partial
335 *Anabax-UB* sequences: *Anabax-UB*01* from toads 7039 and 7092, and *Anabax-UB*02* from
336 toad 6691. Both sequences encode identical α2 and α3 domains but differ by three residues in
337 the α1 domain. *Anabax-UB*01* encodes a signal peptide sequence, but is truncated on the 3'
338 lacking both a TM domain and a stop codon. *Anabax-UB*02* is truncated at both the 5' and 3'
339 ends and encodes only α1, α2 and α3 domains (**Fig 4, Supplemental Table S4,** and
340 **Supplemental Fig S4)**. Regarding *Anabax-UC,* we identified two sequences: *Anabax-UC*01*
341 from toads 6691 and 7092, and *Anabax-UC*02* from toads 7039 and 7092. These sequences
342 encode identical signal peptides, as well as α1 and α2 domains, but lack significant carboxyl-
343 terminal sequences. *Anabax-UC*01* encodes a stop codon 16 residues after the α2 domain,
344 whereas *Anabax-UC*02* lacks a stop codon and may be 3' truncated. (**Fig 4, Supplemental**
345 **Table S4,** and **Supplemental Fig S5)**. Finally, we identified three partial *Anabax-UD*

346 sequences: *Anabax-UD*01* from toads 6691 and 7039, *Anabax-UD*02* from toad 7092 and
347 *Anabax-UD*03* from toad 6691. These sequences encode identical signal peptides, as well as
348 α1 and α2 domains, but differ in the carboxyl-terminus. *Anabax-UD*01* encodes a complete α3
349 domain, but no stop codon. The protein encoded by *Anabax-UD*02* encodes nearly the identical
350 sequence to *Anabax-UD*01* but only includes approximately half of the α3 domain. In contrast,
351 *Anabax-UD*03* encodes what may reflect a partial sequence of an unusual α3 domain that
352 shares little resemblance to the α3 domain of *Anabax-UD*01* (**Fig 4, Supplemental Table S4,**
353 and **Supplemental Fig S6)**.
354
355 *MHC class II sequences*
356 Compared to the MHC class I transcripts, Wyoming toad MHC class II sequences
357 revealed much less diversity displaying only two distinct MHC class IIα chains (*Anabax-DAA*
358 and *Anabax-DBA*) (**Fig. 5**) and two putative haplotypes of a single MHC IIβ chain (*Anabax-*
359 *DAB*01* and *Anabax-DAB*02*) (**Fig. 6** and **Supplemental Table S5**). Two additional, but partial,
360 class IIβ sequences were also identified, but not included in this analysis (**Supplemental Table**
361 **S5**).
362 We found all *Anabax-DAA* transcripts to encode identical full-length proteins between
363 individuals, a finding that was repeated across *Anabax-DBA* transcripts (**Supplemental Table**
364 **S5**). The *Anabax*-DAA and *Anabax*-DBA proteins possess signal peptide sequence, α1, α2 and
365 transmembrane domains (**Supplementary Figure S7**). A one-to-one comparison revealed that
366 the α1 domains from *Anabax*-DAA and *Anabax*-DBA are 52% identical and that the α2 domains
367 are more similar at 68% identity. In our analysis, *Anabax*-DAA is most similar to cane toad
368 *Rhimar*-DAA, whereas *Anabax*-DBA is most similar to *Rhimar*-DCA (**Fig. 5).** In contrast to the
369 homogeneity within *Anabax*-DAA or *Anabax*-DBA transcripts, *Anabax-DAB* transcripts encode
370 full-length proteins that represent two distinct groups: *Anabax*-DAB*01 and *Anabax*-DAB*02 that
371 are collectively most similar to cane toad *Rhimar*-DAB (**Fig. 6**). Both *Anabax*-DAB*01 and

372 *Anabax*-DAB*02 encode identical signal peptide and β1 domains. However, the β2 domains
373 between groups differ at 7 residues (92% identity) and each group possesses distinct
374 transmembrane domains (**Supplementary Fig. S8**). We identified identical *Anabax*-DAB*01
375 coding sequences in all three individuals, while *Anabax*-DAB*02 sequences were only
376 recovered from toads 7039 and 7092 (**Supplementary Table S5**).
377
378 **Discussion**
379
380 Genetic management tools for the Wyoming toad are severely lacking, necessitating
381 breeding facilities to utilize studbooks and mean kinship analyses in hopes of maintaining
382 diversity within the captive populations (Vincent et al. 2015). While recent studies have shown
383 reduced diversity at microsatellite loci (Martin et al. 2019), no studies to date have assessed
384 variation at adaptive loci or genes responsible for immune function that may confer an adaptive
385 advantage to Bd. Our results indicate that Wyoming toads do not exhibit a high level of
386 variability between individuals at TLR or MHC loci. In particular, sequence diversity of TLR and
387 MHC class II transcripts is nearly homogenous between individuals. This sequence similarity
388 highlights the need for future investigations that assess whether Wyoming toad, or even bufonid
389 species in general, have reduced sequence diversity in these core loci of the innate immune
390 system relative to other anurans. In contrast, MHC class I transcripts display enough sequence
391 variability to predict that at least two MHC class I genes are present in the Wyoming toad
392 genome and that each gene likely has two, or possibly three, haplotypes that may influence
393 immune function.
394 The conserved nature of toll-like receptor genes and their role in the innate immune
395 response within species necessitates higher levels of sequence conservation than those
396 exhibited by MHC loci. From our transcriptome analyses, we identified transcripts representing
397 twelve different TLRs (TLR1a, TLR1b, TLR2, TLR3, TLR4, TLR5, TLR8a, TLR8b, TLR12,

398 TLR13, TLR14 and TLR21) (**Fig. 2**). These TLR receptors are also present in *Xenopus* species.
399 However, relative to *Xenopus*, the Wyoming toad is missing TLR7, TLR9 and TLR22 and does
400 not appear to encode any additional TLRs. TLR7 is known to bind single stranded RNA and
401 TLR9 recognizes unmethylated CpG DNA sequences (Akira and Takeda 2004; Vidya et al.
402 2018). TLR22 has been described from amphibians and teleost fishes and studies with teleost
403 TLR22 suggest it recognizes microbial RNA (Samanta et al. 2014; Ji et al. 2019; Du et al. 2019).
404 Further studies of anuran TLR function are needed to better characterize the implications of
405 these gene losses for the Wyoming Toads.

406 Within Wyoming toads, we identified full-length transcripts encoding identical proteins
407 across all individuals for nearly all TLRs. The exceptions to sequence homogeneity comprised
408 TLR2 (7039 encodes a truncated sequence), TLR8a (7039 encodes a different TIR domain),
409 TLR12 (the 7092 TIR domain differs by a single residue, 6691 encodes a truncated ectodomain,
410 and all three individuals vary within the ectodomain at numerous residues) and TLR14 (7039
411 encodes a truncated sequence). Further, TLR8a and TLR8b transcripts identified from Wyoming
412 toad encode identical ectodomains (**Supplemental Table S6**). This result stands in contrast to
413 expectations from common toad and cane toad where these genes are predicted to encode
414 distinctly different ectodomains that presumably bind different PAMPs. We classify Wyoming
415 toad TLR8a and TLR8b as different sequences as their TIR domains are only 89% identical
416 (**Fig. 2** and **Supplemental Table S7**). Although this observation might suggest that the TLR8
417 paralogs in Wyoming toad have not diversified as observed in common toad and cane toad, we
418 cannot exclude the possibility that what we identify as TLR8a and TLR8b from the Wyoming
419 toad are different haplotypes of the same gene, and that Wyoming toad encodes a second TLR
420 gene that was not detected in our transcriptome analyses. Collectively, the loss of three TLRs
421 and lack of diversity between TLR8a and TLR8b ectodomains may impact the ability of the
422 Wyoming toad to mount a successful immune defense against viral pathogens. It is our hope

423 that this classification of Wyoming toad TLRs, combined with sequences from the common toad
424 and cane toad may facilitate future studies on their role in early immune responses to infection.
425 Genes within the MHC are hotspots for polymorphisms and are some of the most
426 diverse regions within the vertebrate genome (Eizaguirre et al. 2012; Radwan et al. 2020).
427 Because of their high level of variability and their importance in adaptive immunity, peptide
428 binding regions of MHC molecules are often targets for selection with heterozygosity typically
429 conveying an advantage, as they are able to present a wider array of peptides (McClelland Erin
430 E. et al. 2003; Sommer 2005; Savage and Zamudio 2011). However, Wyoming toad transcripts
431 identified as MHC class I revealed four distinct groups that were nearly identical between
432 individuals. In general, these groups have different residues across the peptide binding regions
433 for each domain. In instances where their transcripts had both identical and non identical
434 domains (UA α2 domain and UB α1 domain), putative binding regions shared identical amino
435 acid residues except for the α2 domain in UA where the two transcripts had different amino
436 acids at the second peptide binding region (**Fig. 4** and **Supplementary Figures S3** and **S4**).
437 Compared to cane toads, Wyoming toads have a greater number of expressed MHC class I
438 transcripts, however we found only one full length sequence, *Anabax-UA,* that we feel
439 confidently acts as a true MHC class I molecule. Other MHC class I sequences were truncated,
440 which could indicate that these sequences are from genes encoding non-classical or secreted
441 MHCI molecules or indicate sequencing or assembly error. Increased sampling beyond the
442 scope of this project, including genomic analyses and inclusion of additional individuals, would
443 be needed to distinguish between these two alternatives.
444 The three Wyoming toads examined in this study also revealed a reduced MHC class II
445 repertoire relative to their MHC class I sequences, as well as in comparison to other
446 bottlenecked bufonids, such as cane toads (Lillie et al. 2014; Selechnik et al. 2019). We found
447 two class IIα transcripts, *Anabax-DAA* and *Anabax-DBA*, that were present across all individuals
448 with 100% sequence identity. Out of 18 putative peptide binding residues, eight are different

449 between the two transcript classes. Similarly, two class IIβ transcripts, both *Anabax-DAB,* were
450 identified which are likely haplotypes of the same gene. These sequences encode identical β1
451 domains and thus possess identical peptide binding regions. In several amphibian species,
452 specific changes within the peptide binding regions of MHC IIβ (DAB) have been associated
453 with chytridiomycosis susceptibility or tolerance through either immune response regulation or
454 direct interaction with Bd peptides (Richmond et al. 2009; Savage and Zamudio 2011; Bataille et
455 al. 2015; Savage et al. 2020). This linkage between MHC class II and Bd suggests the
456 possibility for strong selective pressures towards specific MHC class II conformations in
457 anurans. Our finding of near homogeneity at MHC class II highlights a significant genotypic
458 challenge facing this species in regard to overall disease pressures. Further studies across
459 larger numbers of individuals from the breeding program are critically needed to investigate
460 whether selective breeding for more diverse MHC class II genotypes is possible within the
461 captive population.

462 A limitation of our study is the small number of individuals that could be used due to the
463 lethal nature of taking samples from immune tissues. In hopes of increasing diversity, toads are
464 now managed as one population rather than three smaller subpopulations (Vincent et al. 2015).
465 While no B strain toads were available for this study, having M (7092) and A (6691 & 7039)
466 toads (see Methods) should provide insights into the overall genetic diversity that formed the
467 basis of the breeding program. Future genomics research evaluating genomic markers that
468 confer an advantage for wild persistence in reintroduced individuals would be warranted.

469 Bottleneck events are not uncommon in amphibian species, and are forecast to increase
470 due to climate change, habitat fragmentation and loss, and the introduction of disease (Allentoft
471 and O'Brien 2010; Pabijan et al. 2020). While the Wyoming toads examined in this study display
472 a low level of variation at immune loci, the ability to recover from a bottleneck relies on more
473 than immunogenetic diversity. A number of highly invasive Anuran species have repeatedly
474 undergone multiple bottleneck events across the world (Beard and Pitt 2005; Ficetola et al.

475 2008; Forti et al. 2017). For example, the Cane toad is one of the world's most successful
476 invasive species of amphibians (Phillips et al. 2007; Brown et al. 2015) yet has a reduced
477 classical MHC Class I repertoire. Additionally, MHC diversity can vary naturally across
478 populations. In amphibian lineages that span Hochstetter's frogs (*Leiopelma hochstetteri*) to
479 crested newts (*Triturus cristatus*) MHC class II diversity varies between populations from high to
480 very little. In these cases, the immunological consequences of such differences remain
481 unknown (Babik et al. 2009; Lillie et al. 2015). In other species, reduced diversity at MHC loci,
482 due to either natural variation or bottlenecking events, can correlate with increased infection and
483 differential survivorship (Belasen et al. 2019; Kosch et al. 2019). This is exemplified by common
484 toads in Sweden, where overall MHC class II diversity increases from north to south, with
485 northern populations showing a higher susceptibility to, and mortality from, disease relative to
486 more diverse southern populations (Thörn et al. 2021). These examples highlight the complexity
487 in correlating diversity of immune loci to the role of disease in the recovery of populations from
488 bottlenecking events. Future studies of the selective pressures placed on the immunome of
489 Wyoming toads are critically needed.

490 As they continue to recover from near extinction, the outlook for Wyoming toads no
491 longer looks as grim as it once did. However, there are still numerous challenges facing the
492 species including the threat of Bd and other pathogens, as well as reduced genetic diversity. We
493 recommend the inclusion of immune associated genetic markers to evaluate genetic variation
494 within captive and wild Wyoming toad populations to improve management decisions. With the
495 devastating effects of infectious diseases like Bd, it has become vital for conservation
496 management practices to incorporate immunogenetics and broader "omics" techniques to better
497 understand the interplay between variation at immune loci and disease outcomes. The results of
498 this study highlight the need for more in depth research on the immunome of not only this
499 species, but also others that are threatened with extinction.
500

501 **Conclusion**

502 Our analyses indicate that overall immunogenetic diversity within the Wyoming toad
503 species is minimal at best, reflecting overall expectations of genomic diversity in studies using
504 microsatellite markers (Martin et al. 2019). While overall genetic diversity may be low, our
505 results provide potential immune gene markers that could be combined with other markers to
506 create a genetic panel to better monitor species-wide diversity. The wide application of
507 immunogenetics in conservation (including studies on diversity, mate selection, and disease
508 tolerance), coupled with greater accessibility for next generation sequencing, underscores the
509 potential for incorporating such assays into the management of species of concern. With more
510 than 40% of amphibians experiencing decline due to disease or anthropogenic pressures
511 (Pabijan et al. 2020), understanding the relationship between core functional genomic regions
512 and species persistence is critical for the future of a diverse Amphibia. As population loss trends
513 continue across the planet, captive breeding programs have become increasingly common
514 (Farquharson et al. 2021) and "omics" techniques may be key tools for understanding host
515 pathogen interactions and guiding management decisions in species of concern.
516

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524 https://commons.wikimedia.org/wiki/File:African_clawed_frogs;_Xenopus_laevis.jpg
525 https://commons.wikimedia.org/wiki/File:Bufo-bufo-erdkroete-maennlich-front.jpg
526 https://commons.wikimedia.org/wiki/File:Cane-toad.jpg

527 https://commons.wikimedia.org/wiki/File:Anaxyrus_baxteri-3.jpg

528

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539

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542

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552

553 Data Availability. Transcriptome sequence data has been archived on NCBI as BioProject

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555 and SRX4501369 (toad 7092); and TSA accession numbers accession numbers

556 GGUS00000000 (toad 6691), GGUR00000000 (toad 7039), GGUQ00000000 (toad 7092).

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558 **Figure legends:**

559

560 **Fig. 1 Wyoming toad chromosomes. a** Chromosomal spread and **b** karyotype of a metaphase
561 preparation of a Wyoming toad (*Anaxyrus baxteri*) 7039. Wyoming toads possess 11 pairs of
562 chromosomes with no evidence of cryptic polyploidy.

563

564 **Fig. 2 Wyoming toad transcriptomes reveal 13 toll-like receptor genes.** Phylogenetic tree
565 comparing TLR TIR domains of three Wyoming toads to those in five other ranid species,
566 including two additional bufonids. Sequences are from Wyoming toad (*Anabax*), common toad
567 (*Bufbuf*), cane toad (*Rhimar*), *Xenopus laevis* (*Xenlae*) and *Xenopus tropicalis* (*Xentro*).
568 Sequence identifiers for Wyoming toad TLRs are provided in **Supplemental Table S3**. If
569 available, GenBank or Xenbase identifiers are included in the figure with sequence names. As
570 TLR LRR ectodomains are typically variable between species, the more conserved TIR domains
571 were employed for phylogenetic analyses (Quiniou et al. 2013; Boudinot et al. 2014; Wcisel et
572 al. 2017). Wyoming toads express TLRs in all six of the major TLR families with identical or
573 nearly identical sequences across the three individuals. Circles at nodes indicate bootstrap
574 support values (BSS) with filled black circles indicating BSS=100, gray circles indicating BSS
575 values equal to or greater than 90 but less than 100, and white circles indicating BSS values
576 greater than 70 but less than 90. Scale bar indicates substitutions over time.

577

578 **Fig. 3 Phylogenetic comparison of Wyoming toad MHC class I sequences to other**
579 **anurans.** Phylogenetic trees comparing Wyoming toad (*Anabax*) MHC class I protein
580 sequences to those of common toad (*Bufbuf*), cane toad (*Rhimar*), *Xenopus laevis* (*Xenlae*) and
581 *Xenopus tropicalis* (*Xentro*). Trees were generated using alignments of (a) contiguous α1-α2-α3
582 domains or by using individual protein domains; (b) α1, (c) α2, and (d) α3. Note that *Bufbuf*
583 "F10-like(b)" is likely a "Q9-like" sequence and inaccurately annotated. Circles at nodes indicate

584 bootstrap support values (BSS) with filled black circles indicating BSS=100, gray circles
585 indicating BSS values equal to or greater than 90 but less than 100, and white circles indicating
586 BSS values greater than 70 but less than 90. Scale bar indicates substitutions over time.
587
588 **Fig. 4 Wyoming toad transcriptomes reveal four distinct groups of MHC class I**
589 **sequences.** Annotated alignments of individual MHC class I (a) α1, (b) α2, and (c) α3 domains
590 from Wyoming toad (*Anabax*) with common toad (*Bufbuf*), cane toad (*Rhimar*), *Xenopus laevis*
591 (*Xenlae*) and *Xenopus tropicalis* (*Xentro*). Asterisks indicate putative peptide binding sites as
592 described by Lillie et al (2014).
593
594 **Fig. 5 Wyoming toad transcriptomes reveal two distinct groups of MHC class IIα**
595 **sequences.** Phylogenetic trees comparing Wyoming toad (*Anabax*) MHC class IIα chain protein
596 sequences to common toad (*Bufbuf*), cane toad (*Rhimar*), *Xenopus laevis* (*Xenlae*) and
597 *Xenopus tropicalis* (*Xentro*). Trees were generated using alignments of (a) contiguous α1-α2
598 domains or by using individual protein domains; (b) α1, and (c) α2. Annotated alignments of
599 individual (d) α1, and (e) α2 domains from Wyoming toad with other anuran species. Asterisks
600 indicate putative binding sites as described by Lillie et al (2016). Circles at nodes indicate
601 bootstrap support values (BSS) with filled black circles indicating BSS=100, gray circles
602 indicating BSS values equal to or greater than 90 but less than 100, and white circles indicating
603 BSS values greater than 70 but less than 90. Scale bar indicates substitutions over time.
604
605 **Fig. 6 Wyoming toad transcriptomes reveal a single group of MHC class IIβ sequences.**
606 Phylogenetic trees comparing Wyoming toad (*Anabax*) MHC class IIβ chain protein sequences
607 to common toad (*Bufbuf*), cane toad (*Rhimar*), *Xenopus laevis* (*Xenlae*) and *Xenopus tropicalis*
608 (*Xentro*). Trees were generated using alignments of (a) contiguous β1-β2 domains or by using
609 individual protein domains; (b) β1, and (c) β2. Annotated alignments of individual (d) β1, and (e)

610 β2 domains from Wyoming toad with other anuran species. Asterisks indicate putative binding
611 sites as described by Lillie et al (2016). Circles at nodes indicate bootstrap support values
612 (BSS) with filled black circles indicating BSS=100, gray circles indicating BSS values equal to or
613 greater than 90 but less than 100, and white circles indicating BSS values greater than 70 but
614 less than 90. Scale bar indicates substitutions over time.

615 **References**

616 Akira S, Takeda K (2004) Toll-like receptor signalling. Nat Rev Immunol 4:499–511.
617 https://doi.org/10.1038/nri1391

618 Allendorf FW, Hohenlohe PA, Luikart G (2010) Genomics and the future of conservation
619 genetics. Nat Rev Genet 11:697–709. https://doi.org/10.1038/nrg2844

620 Allentoft ME, O'Brien J (2010) Global Amphibian Declines, Loss of Genetic Diversity and
621 Fitness: A Review. Diversity 2:47–71. https://doi.org/10.3390/d2010047

622 Altschul SF, Gish W, Miller W, et al (1990) Basic local alignment search tool. J Mol Biol
623 215:403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

624 Babik W, Pabijan M, Arntzen JW, et al (2009) Long-term survival of a urodele amphibian despite
625 depleted major histocompatibility complex variation. Mol Ecol 18:769–781.
626 https://doi.org/10.1111/j.1365-294X.2008.04057.x

627 Bataille A, Cashins SD, Grogan L, et al (2015) Susceptibility of amphibians to chytridiomycosis
628 is associated with MHC class II conformation. Proc Biol Sci. 282(1805): 20143127.
629 https://doi.org/10.1098/rspb.2014.3127

630 Beard KH, Pitt WC (2005) Potential consequences of the coqui frog invasion in Hawaii. Divers
631 Distrib 11:427–433. https://doi.org/10.1111/j.1366-9516.2005.00178.x

632 Belasen AM, Bletz MC, Leite D da S, et al (2019) Long-Term Habitat Fragmentation Is
633 Associated With Reduced MHC IIB Diversity and Increased Infections in Amphibian Hosts.
634 Frontiers in Ecology and Evolution 6:236. https://doi.org/10.3389/fevo.2018.00236

635 Berger L, Speare R, Daszak P, et al (1998) Chytridiomycosis causes amphibian mortality
636 associated with population declines in the rain forests of Australia and Central America.
637 Proc Natl Acad Sci U S A 95:9031–9036

638 Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence
639 data. Bioinformatics 30:2114–2120. https://doi.org/10.1093/bioinformatics/btu170

640 Boudinot P, Zou J, Ota T, et al (2014) A tetrapod-like repertoire of innate immune receptors and
641 effectors for coelacanths. J Exp Zool B Mol Dev Evol 322:415–437.
642 https://doi.org/10.1002/jez.b.22559

643 Boyle DG, Boyle DB, Olsen V, et al (2004) Rapid quantitative detection of chytridiomycosis
644 (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR
645 assay. Dis Aquat Organ 60:141–148. https://doi.org/10.3354/dao060141

646 Breen M, Hitte C, Lorentzen TD, et al (2004) An integrated 4249 marker FISH/RH map of the
647 canine genome. BMC Genomics 5:65. https://doi.org/10.1186/1471-2164-5-65

648 Brown GP, Kelehear C, Shilton CM, et al (2015) Stress and immunity at the invasion front: a
649 comparison across cane toad (Rhinella marina) populations. Biol J Linn Soc Lond 116:748–
650 760. https://doi.org/10.1111/bij.12623

651 Bryant DM, Johnson K, DiTommaso T, et al (2017) A Tissue-Mapped Axolotl De Novo

652 Transcriptome Enables Identification of Limb Regeneration Factors. Cell Rep 18:762–776.
653 https://doi.org/10.1016/j.celrep.2016.12.063

654 Burton EC, Gray MJ, Schmutzer AC, Miller DL (2009) Differential responses of
655 postmetamorphic amphibians to cattle grazing in wetlands. J Wildl Manage 73:269–277.
656 https://doi.org/10.2193/2007-562

657 Chen G, Robert J (2011) Antiviral immunity in amphibians. Viruses 3:2065–2086.
658 https://doi.org/10.3390/v3112065

659 Conde DA, Staerk J, Colchero F, et al (2019) Data gaps and opportunities for comparative and
660 conservation biology. Proc Natl Acad Sci U S A 116:9658–9664.
661 https://doi.org/10.1073/pnas.1816367116

662 Daszak P, Cunningham AA, Hyatt AD (2003) Infectious disease and amphibian population
663 declines. Divers Distrib 9:141–150. https://doi.org/10.1046/j.1472-4642.2003.00016.x

664 Dickerson KK, Hooper MJ, Huang T, Allen M (2003) Determination of Malathion Aerial Drift and
665 Associated Effects to the Endangered Wyoming Toad (Bufo baxteri) Using Surrogate
666 Woodhouse's Toads (Bufo woodhousii) at Mortenson and Hutton Lake National Wildlife
667 Refuges and Potential Reintroduction Sites. In: Multiple Stressor Effects in Relation to
668 Declining Amphibian Populations. ASTM STP 1443. G. Linder, S. Krest, D. Sparling, and E.
669 Little, Eds., American Society for Testing and Materials, West Conshohocken, PA

670 Dirscherl H, McConnell SC, Yoder JA, de Jong JLO (2014) The MHC class I genes of zebrafish.
671 Dev Comp Immunol 46:11–23. https://doi.org/10.1016/j.dci.2014.02.018

672 Dreitz VJ (2006) Issues in species recovery: an example based on the Wyoming toad.
673 BioScience 56:765–771. https://doi.org/10.1641/0006-3568(2006)56[765:IISRAE]2.0.CO;2

674 Du X, Wu J, Li Y, et al (2019) Multiple subtypes of TLR22 molecule from Schizothorax prenanti
675 present the functional diversity in ligand recognition and signal activation. Fish Shellfish
676 Immunol 93:986–996. https://doi.org/10.1016/j.fsi.2019.08.042

677 Eizaguirre C, Lenz TL, Kalbe M, Milinski M (2012) Rapid and adaptive evolution of MHC genes
678 under parasite selection in experimental vertebrate populations. Nat Commun 3:621.
679 https://doi.org/10.1038/ncomms1632

680 Ellison AR, Tunstall T, DiRenzo GV, et al (2014) More than skin deep: functional genomic basis
681 for resistance to amphibian chytridiomycosis. Genome Biol Evol 7:286–298.
682 https://doi.org/10.1093/gbe/evu285

683 Farquharson KA, Hogg CJ, Grueber CE (2021) Offspring survival changes over generations of
684 captive breeding. Nat Commun 12:3045. https://doi.org/10.1038/s41467-021-22631-0

685 Ficetola GF, Bonin A, Miaud C (2008) Population genetics reveals origin and number of
686 founders in a biological invasion. Mol Ecol 17:773–782. https://doi.org/10.1111/j.1365-
687 294X.2007.03622.x

688 Forti LR, Becker CG, Tacioli L, et al (2017) Perspectives on invasive amphibians in Brazil. PLoS
689 One 12:e0184703. https://doi.org/10.1371/journal.pone.0184703

690 Fortriede JD, Pells TJ, Chu S, et al (2020) Xenbase: deep integration of GEO & SRA RNA-seq

691 and ChIP-seq data in a model organism database. Nucleic Acids Res 48:D776–D782.
692 https://doi.org/10.1093/nar/gkz933

693 Fulton JE, Berres ME, Kantanen J, Honkatukia M (2017) MHC-B variability within the Finnish
694 Landrace chicken conservation program. Poult Sci 96:3026–3030.
695 https://doi.org/10.3382/ps/pex102

696 Grabherr MG, Haas BJ, Yassour M, et al (2011) Full-length transcriptome assembly from RNA-
697 Seq data without a reference genome. Nat Biotechnol 29:644–652.
698 https://doi.org/10.1038/nbt.1883

699 Gregory TR (2021) Animal Genome Size Database. http://www.genomesize.com. Accessed 3
700 Nov 2021

701 Grogan KE, Sauther ML, Cuozzo FP, Drea CM (2017) Genetic wealth, population health: Major
702 histocompatibility complex variation in captive and wild ring-tailed lemurs (Lemur catta).
703 Ecol Evol 7:7638–7649. https://doi.org/10.1002/ece3.3317

704 Haver M, Le Roux G, Friesen J, et al (2022) The role of abiotic variables in an emerging global
705 amphibian fungal disease in mountains. Sci Total Environ. 815:152735.
706 https://doi.org/10.1016/j.scitotenv.2021.152735

707 Hof C, Araújo MB, Jetz W, Rahbek C (2011) Additive threats from pathogens, climate and land-
708 use change for global amphibian diversity. Nature 480:516–519.
709 https://doi.org/10.1038/nature10650

710 Hornyak V (2012) The Wyoming Toad: Partnerships for Recovery. World Association of Zoos
711 and Aquariums Magazine 366:33–35

712 Ishii A, Kawasaki M, Matsumoto M, et al (2007) Phylogenetic and expression analysis of
713 amphibian Xenopus Toll-like receptors. Immunogenetics 59:281–293.
714 https://doi.org/10.1007/s00251-007-0193-y

715 Jennings M, Anderson A (1997) The Wyoming Toad. Endangered Species Bulletin 22:16–17

716 Jennings M, Beiswenger R, Corn PS, et al (2001) Population and habitat viability assessment
717 for the Wyoming toad (Bufo baxteri): Final workshop report. Conservation Breeding
718 Specialist Group, Apple Valley, MN

719 Ji J, Liao Z, Rao Y, et al (2019) Thoroughly Remold the Localization and Signaling Pathway of
720 TLR22. Front Immunol 10:3003. https://doi.org/10.3389/fimmu.2019.03003

721 Khan I, Maldonado E, Silva L, et al (2019) The vertebrate TLR supergene family evolved
722 dynamically by gene gain/loss and positive selection revealing a host-pathogen arms race
723 in birds. Diversity 11.: https://doi.org/10.3390/d11080131

724 Klein J, Bontrop RE, Dawkins RL, et al (1990) Nomenclature for the major histocompatibility
725 complexes of different species: a proposal. Immunogenetics 31:217–219.
726 https://doi.org/10.1007/BF00204890

727 Kosch TA, Silva CNS, Brannelly LA, et al (2019) Genetic potential for disease resistance in
728 critically endangered amphibians decimated by chytridiomycosis. Anim Conserv 22:238–
729 250. https://doi.org/10.1111/acv.12459

730 Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods
731 9:357–359. https://doi.org/10.1038/nmeth.1923

732 Letunic I, Khedkar S, Bork P (2021) SMART: recent updates, new developments and status in
733 2020. Nucleic Acids Research 49:D458–D460

734 Lillie M, Cui J, Shine R, Belov K (2016) Molecular characterization of MHC class II in the
735 Australian invasive cane toad reveals multiple splice variants. Immunogenetics 68:449–
736 460. https://doi.org/10.1007/s00251-016-0919-9

737 Lillie M, Grueber CE, Sutton JT, et al (2015) Selection on MHC class II supertypes in the New
738 Zealand endemic Hochstetter's frog. BMC Evol Biol 15:63. https://doi.org/10.1186/s12862-
739 015-0342-0

740 Lillie M, Shine R, Belov K (2014) Characterisation of major histocompatibility complex class I in
741 the Australian cane toad, Rhinella marina. PLoS One 9.:
742 https://doi.org/10.1371/journal.pone.0102824

743 Lips KR (2016) Overview of chytrid emergence and impacts on amphibians. Philos Trans R Soc
744 Lond B Biol Sci 371.: https://doi.org/10.1098/rstb.2015.0465

745 Lips KR, Brem F, Brenes R, et al (2006) From The Cover: Emerging infectious disease and the
746 loss of biodiversity in a Neotropical amphibian community. Proceedings of the National
747 Academy of Sciences 103:3165–3170. https://doi.org/10.1073/pnas.0506889103

748 Manni M, Berkeley MR, Seppey M, et al (2021) BUSCO Update: Novel and Streamlined
749 Workflows along with Broader and Deeper Phylogenetic Coverage for Scoring of
750 Eukaryotic, Prokaryotic, and Viral Genomes. Mol Biol Evol 38:4647–4654.
751 https://doi.org/10.1093/molbev/msab199

752 Martin RM, Meador H, Bender L, Hopper L (2019) Isolation and characterization of 27 novel
753 microsatellite loci in critically endangered Wyoming toad. Journal of Fish and Wildlife
754 Management 10:563–566. https://doi.org/10.3996/042019-JFWM-029

755 McClelland Erin E., Penn Dustin J., Potts Wayne K. (2003) Major Histocompatibility Complex
756 Heterozygote Superiority during Coinfection. Infect Immun 71:2079–2086.
757 https://doi.org/10.1128/IAI.71.4.2079-2086.2003

758 Minh BQ, Schmidt HA, Chernomor O, et al (2020) IQ-TREE 2: New Models and Efficient
759 Methods for Phylogenetic Inference in the Genomic Era. Mol Biol Evol 37:1530–1534.
760 https://doi.org/10.1093/molbev/msaa015

761 Morgan M, Falcon S, Gentleman R (2018) GSEABase: Gene set enrichment data structures
762 and methods. R package version 1:

763 Nie L, Cai S-Y, Shao J-Z, Chen J (2018) Toll-Like Receptors, Associated Biological Roles, and
764 Signaling Networks in Non-Mammals. Front Immunol 9:1523.
765 https://doi.org/10.3389/fimmu.2018.01523

766 Pabijan M, Palomar G, Antunes B, et al (2020) Evolutionary principles guiding amphibian
767 conservation. Evol Appl 13:857–878. https://doi.org/10.1111/eva.12940

768 Palmer CV (2018) Immunity and the coral crisis. Commun Biol 1:91.

https://doi.org/10.1038/s42003-018-0097-4

Phillips BL, Brown GP, Greenlees M, et al (2007) Rapid expansion of the cane toad (Bufo marinus) invasion front in tropical Australia. Austral Ecol 32:169–176. https://doi.org/10.1111/j.1442-9993.2007.01664.x

Polasik JS, Murphy MA, Abbott T, Vincent K (2016) Factors limiting early life stage survival and growth during endangered Wyoming toad reintroductions: Wyoming Toad Early Life Stage Limits. Jour Wild Mgmt 80:540–552. https://doi.org/10.1002/jwmg.1031

Pounds JA, Bustamante MR, Coloma LA, et al (2006) Widespread amphibian extinctions from epidemic disease driven by global warming. Nature 439:161–167. https://doi.org/10.1038/nature04246

Priyam A, Woodcroft BJ, Rai V, et al (2019) Sequenceserver: A Modern Graphical User Interface for Custom BLAST Databases. Mol Biol Evol 36:2922–2924. https://doi.org/10.1093/molbev/msz185

Punta M, Coggill PC, Eberhardt RY, et al (2012) The Pfam protein families database. Nucleic Acids Res 40:D290–301. https://doi.org/10.1093/nar/gkr1065

Quiniou SMA, Boudinot P, Bengtén E (2013) Comprehensive survey and genomic characterization of Toll-like receptors (TLRs) in channel catfish, Ictalurus punctatus: identification of novel fish TLRs. Immunogenetics 65:511–530. https://doi.org/10.1007/s00251-013-0694-9

Radwan J, Babik W, Kaufman J, et al (2020) Advances in the Evolutionary Understanding of MHC Polymorphism. Trends Genet 36:298–311. https://doi.org/10.1016/j.tig.2020.01.008

Richmond JQ, Savage AE, Zamudio KR, Rosenblum EB (2009) Toward immunogenetic studies of amphibian chytridiomycosis: Linking innate and acquired immunity. Bioscience 59:311–320. https://doi.org/10.1525/bio.2009.59.4.9

Roach JC, Glusman G, Rowen L, et al (2005) The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci U S A 102:9577–9582. https://doi.org/10.1073/pnas.0502272102

Rohr JR, Raffel TR, Blaustein AR, et al (2013) Using physiology to understand climate-driven changes in disease and their implications for conservation. Conserv Physiol 1:cot022. https://doi.org/10.1093/conphys/cot022

Russell T, Lisovski S, Olsson M, et al (2018) MHC diversity and female age underpin reproductive success in an Australian icon; the Tasmanian Devil. Sci Rep 8:4175. https://doi.org/10.1038/s41598-018-20934-9

Samanta M, Swain B, Basu M, et al (2014) Toll-like receptor 22 in Labeo rohita: molecular cloning, characterization, 3D modeling, and expression analysis following ligands stimulation and bacterial infection. Appl Biochem Biotechnol 174:309–327. https://doi.org/10.1007/s12010-014-1058-0

Savage AE, Gratwicke B, Hope K, et al (2020) Sustained immune activation is associated with susceptibility to the amphibian chytrid fungus. Mol Ecol 29:2889–2903. https://doi.org/10.1111/mec.15533

808 Savage AE, Zamudio KR (2011) MHC genotypes associate with resistance to a frog-killing
809 fungus. Proc Natl Acad Sci U S A 108:16705–16710.
810 https://doi.org/10.1073/pnas.1106893108

811 Schenekar T, Weiss S (2017) Selection and genetic drift in captive versus wild populations: an
812 assessment of neutral and adaptive (MHC-linked) genetic variation in wild and hatchery
813 brown trout (Salmo trutta) populations. Conserv Genet 18:1011–1022.
814 https://doi.org/10.1007/s10592-017-0949-3

815 Selechnik D, Richardson MF, Shine R, et al (2019) Increased Adaptive Variation Despite
816 Reduced Overall Genetic Diversity in a Rapidly Adapting Invader. Front Genet 10:1221.
817 https://doi.org/10.3389/fgene.2019.01221

818 Sievers F, Higgins DG (2014) Clustal Omega, accurate alignment of very large numbers of
819 sequences. Methods Mol Biol 1079:105–116. https://doi.org/10.1007/978-1-62703-646-7_6

820 Simão FA, Waterhouse RM, Ioannidis P, et al (2015) BUSCO: assessing genome assembly and
821 annotation completeness with single-copy orthologs. Bioinformatics 31:3210–3212

822 Smallbone W, Ellison A, Poulton S, et al (2021) Depletion of MHC supertype during
823 domestication can compromise immunocompetence. Mol Ecol 30:736–746.
824 https://doi.org/10.1111/mec.15763

825 Sommer S (2005) The importance of immune gene variability (MHC) in evolutionary ecology
826 and conservation. Front Zool 2:16. https://doi.org/10.1186/1742-9994-2-16

827 St Leger RJ (2021) Insects and their pathogens in a changing climate. J Invertebr Pathol
828 184:107644. https://doi.org/10.1016/j.jip.2021.107644

829 Thörn F, Rödin-Mörch P, Cortazar-Chinarro M, et al (2021) The effects of drift and selection on
830 latitudinal genetic variation in Scandinavian common toads (Bufo bufo) following postglacial
831 recolonisation. Heredity 126:656–667. https://doi.org/10.1038/s41437-020-00400-x

832 Varga JFA, Bui-Marinos MP, Katzenback BA (2018) Frog Skin Innate Immune Defences:
833 Sensing and Surviving Pathogens. Front Immunol 9:3128.
834 https://doi.org/10.3389/fimmu.2018.03128

835 Vidya MK, Kumar VG, Sejian V, et al (2018) Toll-like receptors: Significance, ligands, signaling
836 pathways, and functions in mammals. Int Rev Immunol 37:20–36.
837 https://doi.org/10.1080/08830185.2017.1380200

838 Vincent K, Abbott T, Wyoming Toad Recovery Team (2015) Wyoming toad (Bufo hemiophrys
839 baxteri now known as Anaxyrus baxteri) revised recovery plan. US Fish and Wildlife
840 Service, Cheyenne, WY

841 Wcisel DJ, Ota T, Litman GW, Yoder JA (2017) Spotted Gar and the Evolution of Innate
842 Immune Receptors. J Exp Zool B Mol Dev Evol 328:666–684.
843 https://doi.org/10.1002/jez.b.22738

844 Wilkinson L (2011) Ggplot2: Elegant graphics for data analysis by WICKHAM, H. Biometrics
845 67:678–679. https://doi.org/10.1111/j.1541-0420.2011.01616.x

846 Woodhams DC, Bosch J, Briggs CJ, et al (2011) Mitigating amphibian disease: strategies to

847 maintain wild populations and control chytridiomycosis. Front Zool 8:8.
848 https://doi.org/10.1186/1742-9994-8-8

849 Zdobnov EM, Tegenfeldt F, Kuznetsov D, et al (2017) OrthoDB v9.1: cataloging evolutionary
850 and functional annotations for animal, fungal, plant, archaeal, bacterial and viral orthologs.
851 Nucleic Acids Res 45:D744–D749. https://doi.org/10.1093/nar/gkw1119

852

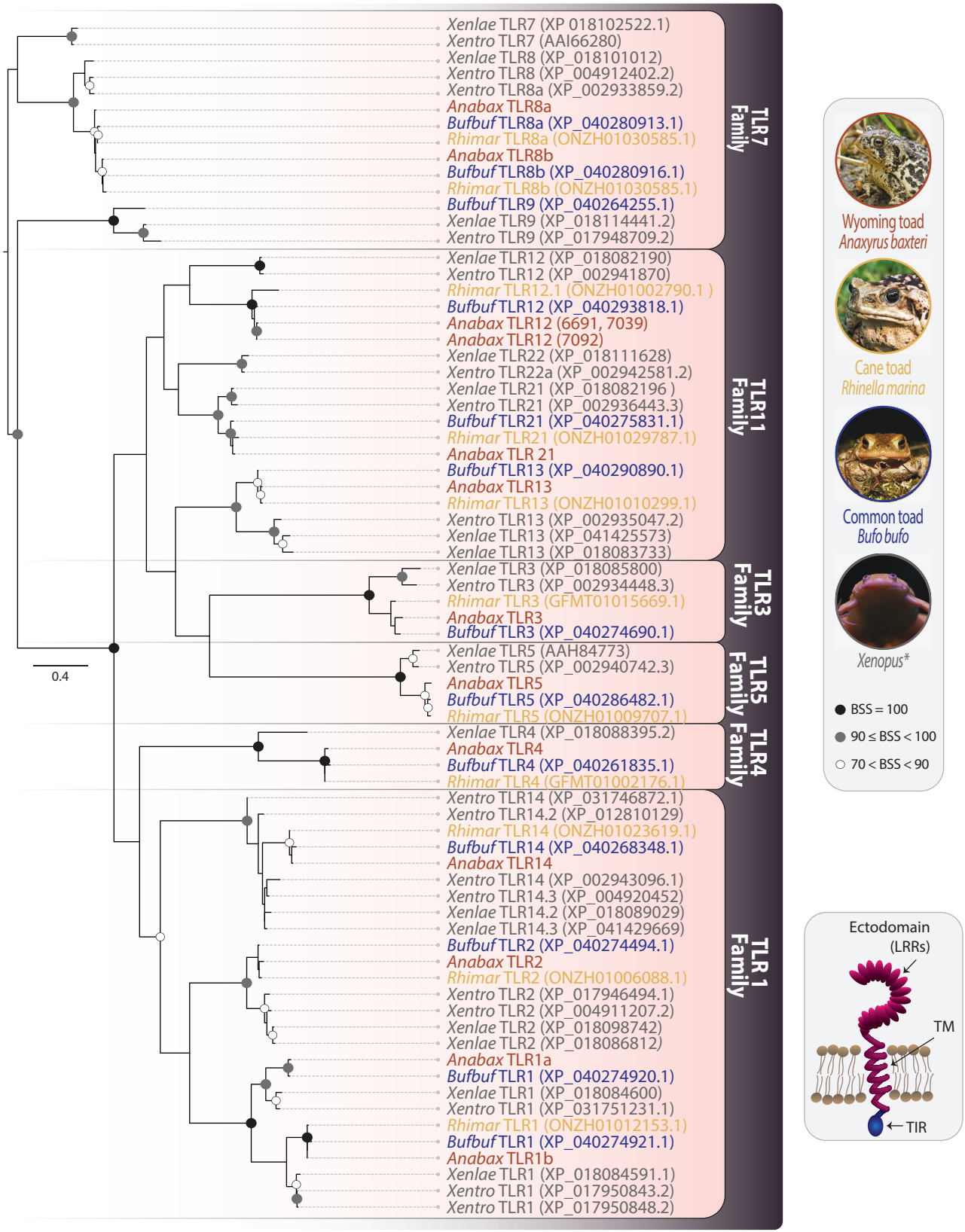
853

854

855

a

b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Xentro TLR7 (AAI66280)
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Anabax TLR8a
Bufbuf TLR8a (XP_040280913.1)
Rhimar TLR8a (ONZH01030585.1)
Anabax TLR8b
Bufbuf TLR8b (XP_040280916.1)
Rhimar TLR8b (ONZH01030585.1)
Bufbuf TLR9 (XP_040264255.1)
Xenlae TLR9 (XP_018114441.2)
Xentro TLR9 (XP_017948709.2)
TLR7 Family
Xenlae TLR12 (XP_018082190)
Xentro TLR12 (XP_002941870)
Rhimar TLR12.1 (ONZH01002790.1)
Bufbuf TLR12 (XP_040293818.1)
Anabax TLR12 (6691, 7039)
Anabax TLR12 (7092)
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Xentro TLR22a (XP_002942581.2)
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Xentro TLR21 (XP_002936443.3)
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Rhimar TLR21 (ONZH01029787.1)
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Bufbuf TLR13 (XP_040290890.1)
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Rhimar TLR13 (ONZH01010299.1)
Xentro TLR13 (XP_002935047.2)
Xenlae TLR13 (XP_041425573)
Xenlae TLR13 (XP_018083733)
TLR11 Family
Xenlae TLR3 (XP_018085800)
Xentro TLR3 (XP_002934448.3)
Rhimar TLR3 (GFMT01015669.1)
Anabax TLR3
Bufbuf TLR3 (XP_040274690.1)
TLR3 Family
Xenlae TLR5 (AAH84773)
Xentro TLR5 (XP_002940742.3)
Anabax TLR5
Bufbuf TLR5 (XP_040286482.1)
Rhimar TLR5 (ONZH01009707.1)
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Anabax TLR4
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TLR4 Family
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Rhimar TLR14 (ONZH01023619.1)
Bufbuf TLR14 (XP_040268348.1)
Anabax TLR14
Xentro TLR14 (XP_002943096.1)
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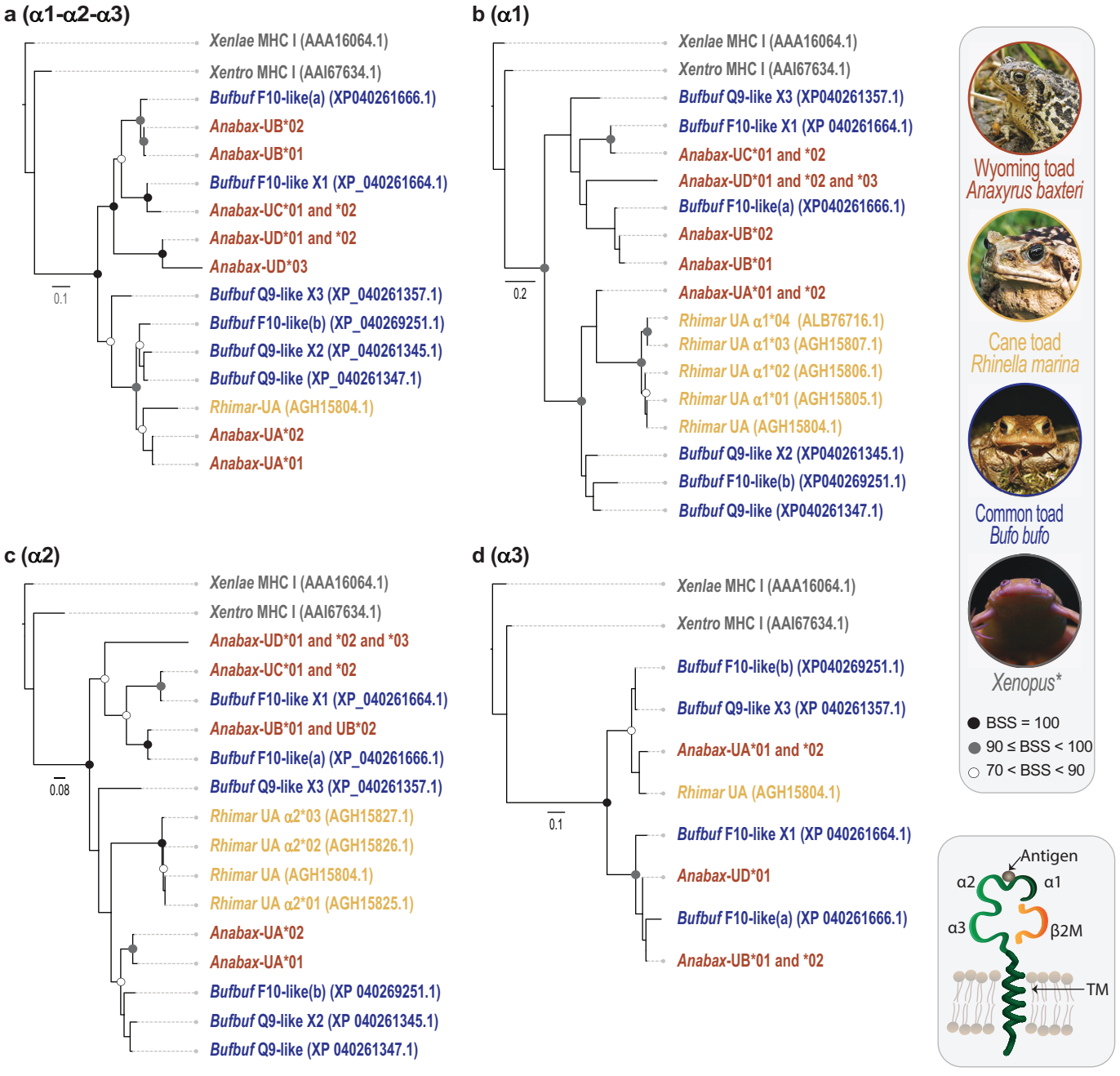
Xenlae TLR14.2 (XP_018089029)
Xenlae TLR14.3 (XP_041429669)
Bufbuf TLR2 (XP_040274494.1)
Anabax TLR2
Rhimar TLR2 (ONZH01006088.1)
Xentro TLR2 (XP_017946494.1)
Xentro TLR2 (XP_004911207.2)
Xenlae TLR2 (XP_018098742)
Xenlae TLR2 (XP_018086812)
Anabax TLR1a
Bufbuf TLR1 (XP_040274920.1)
Xenlae TLR1 (XP_018084600)
Xentro TLR1 (XP_031751231.1)
Rhimar TLR1 (ONZH01012153.1)
Bufbuf TLR1 (XP_040274921.1)
Anabax TLR1b
Xenlae TLR1 (XP_018084591.1)
Xentro TLR1 (XP_017950843.2)
Xentro TLR1 (XP_017950848.2)
TLR 1 Family
0.4
Wyoming toad
Anaxyrus baxteri
Cane toad
Rhinella marina
Common toad
Bufo bufo
Xenopus*
BSS = 100
90 ≤ BSS < 100
70 < BSS < 90
Ectodomain
(LRRs)
TM
TIR


a (α1-α2-α3)
Xenlae MHC I (AAA16064.1)
Xentro MHC I (AAI67634.1)
Bufbuf F10-like(a) (XP040261666.1)
Anabax-UB*02
Anabax-UB*01
Bufbuf F10-like X1 (XP_040261664.1)
Anabax-UC*01 and *02
Anabax-UD*01 and *02
Anabax-UD*03
Bufbuf Q9-like X3 (XP_040261357.1)
Bufbuf F10-like(b) (XP_040269251.1)
Bufbuf Q9-like X2 (XP_040261345.1)
Bufbuf Q9-like (XP_040261347.1)
Rhimar-UA (AGH15804.1)
Anabax-UA*02
Anabax-UA*01
0.1
b (α1)
Xenlae MHC I (AAA16064.1)
Xentro MHC I (AAI67634.1)
Bufbuf Q9-like X3 (XP040261357.1)
Bufbuf F10-like X1 (XP 040261664.1)
Anabax-UC*01 and *02
Anabax-UD*01 and *02 and *03
Bufbuf F10-like(a) (XP040261666.1)
Anabax-UB*02
Anabax-UB*01
Anabax-UA*01 and *02
Rhimar UA α1*04 (ALB76716.1)
Rhimar UA α1*03 (AGH15807.1)
Rhimar UA α1*02 (AGH15806.1)
Rhimar UA α1*01 (AGH15805.1)
Rhimar UA (AGH15804.1)
Bufbuf Q9-like X2 (XP040261345.1)
Bufbuf F10-like(b) (XP040269251.1)
Bufbuf Q9-like (XP040261347.1)
0.2
Wyoming toad
Anaxyrus baxteri
Cane toad
Rhinella marina
Common toad
Bufo bufo
Xenopus*
BSS = 100
90 ≤ BSS < 100
70 < BSS < 90
c (α2)
Xenlae MHC I (AAA16064.1)
Xentro MHC I (AAI67634.1)
Anabax-UD*01 and *02 and *03
Anabax-UC*01 and *02
Bufbuf F10-like X1 (XP_040261664.1)
Anabax-UB*01 and UB*02
Bufbuf F10-like(a) (XP_040261666.1)
Bufbuf Q9-like X3 (XP_040261357.1)
Rhimar UA α2*03 (AGH15827.1)
Rhimar UA α2*02 (AGH15826.1)
Rhimar UA (AGH15804.1)
Rhimar UA α2*01 (AGH15825.1)
Anabax-UA*02
Anabax-UA*01
Bufbuf F10-like(b) (XP 040269251.1)
Bufbuf Q9-like X2 (XP 040261345.1)
Bufbuf Q9-like (XP 040261347.1)
0.08
d (α3)
Xenlae MHC I (AAA16064.1)
Xentro MHC I (AAI67634.1)
Bufbuf F10-like(b) (XP040269251.1)
Bufbuf Q9-like X3 (XP 040261357.1)
Anabax-UA*01 and *02
Rhimar UA (AGH15804.1)
Bufbuf F10-like X1 (XP 040261664.1)
Anabax-UD*01
Bufbuf F10-like(a) (XP 040261666.1)
Anabax-UB*01 and *02
0.1
Antigen
α2
α1
α3
β2M
TM

a (α1)

```
Anabax-UA*01 and *02      DTHSLRYYMTGVSAPGSGLPEYSEVGYVDDREIVNYNSESGRMEPKVKWMEK-VDPGYWERNTQIAKGNPAVNKHNVRTLMSRFNQTG
Anabax-UB*01              -SHSLRYYYTGVSAPGSGLPEFSIVGYMDDQQTELYNSDIGKCIPVATWVRKE-RPEQWLKKTLTSKANPALFKHEVKIVMKRFNHTE
Anabax-UB*02              DSHSLYYCYTGVTAPGSGLPEFSIVGYMDDQQTELYNSDIGKCIPVATWVRKE-RPEQWLKKTLTSKANPALFKHEVKIVMKRFNHTE
Anabax-UC*01 and *02      DSHSLRYYSIGVSAPGSGLPEFSIIGYVDDQQIELYSGDTGRSVPVAPWLSRNVGLEHWERRTRISKEYPALFKHEVKAAVKRFNHTG
Anabax-UD*01 and *02 and *03 DSHSLYYCYTGVTAPGSGLPEFSVVGYMDGQQVELYTSDIGRSVPVAHWLKEKEDSKFWDELTRIRQYSETFFRNELKIAVKRFNHTK

Rhimar-UA                 DSHSLRYYVTGVSAPGSGLPEYSEVGYVDDQEIANYNTESRNYRPKVKWMGK-METDYWGRETQIARQAPAVNRHNMKTLMSRFNQTE

Bufbuf Q9-like            DSHSLRYYCTGVSAPGSGLPEFSIVGYVDDREIVNYNSESGRAKPKVKWMEK-VDPGYWEDNTQRAKGDPVSFRHSVRTVMSRFNQTG
Bufbuf Q9-like X2         DSHSLRYYYTAVSALGSRLPEFSIVGYVDNREIVNYNSESGRAKPKVKWMEK-VDPGYWEEQTQSAKGDPAVSRHNVRTAMSRFNQTG
Bufbuf Q9-like X3         DSHSLRYYYTGVSAPGSGLPEFSIVGYVDDQQIDLYNSDIKKNIPVAPWMKK--DPEYWRRETDIAKQTPPLIKHDVKTAMRRFNQTE
Bufbuf F10-like(b)        DSHSLRYYCTGVTAPGSKLPEYSSVGYLDDREIVNYNSESRKYRPKVKWMEK-VDPGYWKEQTRIAKGNPASFRESVRTVMSRFNQTG
Bufbuf F10-like(a)        DSHSLRYPYTGVSAPGSGLPEFSIVGYMDDQQTELYNSDIGKCVPVATWVRKE-RPEQWLKRTLISKANPALFKHEVKIVMKRFNHTE
Bufbuf F10-like X1        DSHSLRYYSIGVSAPGSGLPEFSIVGYVDDQQIELYSGDIGRSVPVAPWMSRNVGLEHWERRTRISKEYPALFKHEVKAAVKRFNHTG

Xenlae MHC I              GSHSLRYYYTAVSDRAFGLPEFSTVGYVDDTQIERYSSDTGRDEPATQWMKQKEGPEYWERETQKSKGIPATFKHNVKVAMDRFNQST
Xentro MHC I              GSHSLRYYYTGVSDRAFGLPEFSIVGYVDETQIVRYSSDNGRAEPATQWMKQNEGPEYWDRQTQNSKGTPPVYKHNVKVAMDRFNQTS
                                   *                          *  *                    **  ***  **   *   **   **
```

b (α2)

```
Anabax-UA*01              GFHIVQAMYGCERRDDGGITVYDQHGYDGGEFMSLDTQTWTPIPTMSQAQITAQRWNSPEEQWGQRYKNYLEIECKDWLQKYVENGREDLERR
Anabax-UA*02              GFHIVQWMYGCERIDDGGITGYDQHGYDGGEFMSLDTQTWTVIPTMSQAQITAQRWNSPEEQWGQRYKNYLEIECKDWLQKYVENGREDLERR
Anabax-UB*01 and *02      GLHFAQVMHSCELKDDGSIVSYEEFRYDGREYMYLDIKTGLEIPTMAEAQITTQRWNSPDVRAGQRIRNYLANECIDRLRRYVVYGREDLERR
Anabax-UC*01 and *02      GFHFVQVMHNCEMRDDGSTTCHQEYRYDGEEYMYLDIKSALENPTMAEAQIITQRWNSPDIRKGEREKNYLESKCIERLKKYLEFGREDLERR
Anabax-UD*01 and *02 and *03 GFHYVQAMLGCELRDDGSTIGFNQYANDGSEFLHLDLQTKTPIPTMAEAQIITQRWNSWESGIRERVKD-NDIVCINRLNRYLKHGRQHLERR

Rhimar-UA                 GIHILQRMYGCELRDDGSIKGYEQLGYDGEEFMSLDTQTWTPIPTMPQAQISTERLNNAG--EGGSQKNYLEDKCVYWLNKYVEHGREDLERR

Bufbuf Q9-like            GFHIVQWMYGCERRDDGSVTGYDQHGYDGGEFMSLDTQTWTYIPTMSQAQISAQRWNSREEQVVERHKNYLENICIDWLQKHVENGREDLERR
Bufbuf Q9-like X2         GFHIVQWMYGCERRDDGSVTGYDQYGYDGGEFLSLDTQTWTYIPTMSQAQITAQKWNSREVQVVERQKNYLENICIDWLQKHVENGREDLERR
Bufbuf Q9-like X3         GFHFYQVMYGCERRDDGSITGYEQHGYDGRELIHLDTQTWTFIPTLSQAQITTEKINSPLVQAGMRSKHYLEDECITALKEYLASGREDLERR
Bufbuf F10-like(b)        GFHIVQLMYGCERRDDGSVTGYSQYGYDGGEFMSLDTQTWTYIPTMSQAQITAQRWNSPEAQMGERTKNYLETECIDWLQKHVENGREDLERR
Bufbuf F10-like(a)        GFHFAQVMHSCELKDDGSIVNYEEFRYDGREYMYLDIKTGLFISTMAEAQITTQRWNSPDVRAGERIRNYLANECIDRLRRYVVYGREDLERR
Bufbuf F10-like X1        GFHFVQVMHNCEMRDDGSTIGHQEYRYDGEEYMYLDIKTALENPTMAEAQIITQRWNSPDIRKGEREKNFLESKCIERLKKYLEFGREDLERR

Xenlae MHC I              GTHMVQWMYGCELGDDGSTRGYEQHVYDGREFFALDTEEWVYVPSVREAQLTTQKWNSPEVNAPERNKNYLQNICIEGLKRYLSYGQAELERR
Xentro MHC I              GTHSLQYMYGCELREDGSTRGYEQFGYDGRELMALDTERWRYVPSMREAQLSTQKWNSPEVNEPERFNYLENICIPALKRYLGYGQAELERR
                               *  *                  *  *                                  **  **    *    *
```

c (α3)

```
Anabax-UA*01 and *02      VQPQVKVSGQKKDDAMMLHCQVYGFHPRPVHVKWMKNK-DDVHSYETTHTLPNPDGTYQIRVSAEVIPKEGESYSCYVDHSSIKEPLNIVWE
Anabax-UB*01 and *02      VQPGVKVTGRESGEITKLHCLVYGFHPRAVDVKWMKNGIDEIPSYETTHVLPNPDGTYQIRVSVEVIPKEGESYSCY----------
Anabax-UD*01              VQPGVKVTGRESGEVTKLHCHVYGFHPRAVDVKWMKNGIDEIPSYETTHVLPNPDGTYQIRVSVEVIPKEGESYS----------

Rhimar-UA                 VQPQVKVSGQKKDDAMMLHCQVYGFHPRPVHVKWMKNE-DDVPSYESTHTLPNPDGTYQIRVSAEVIPEEGDSYSCYVDHSSIGEPLNVVWE

Bufbuf Q9 -like           VQPQVKVSGQKKGDTMMLHCQVYGFHPRAVHVKWMKNG-DDVPDYETTHTLPNPDGTYQIRVSAEVIPKEGESYSCYVDHSSIGEPLNVVWE
Bufbuf Q9-like X2         VQPQVKVSGQKKGDTMMLHCQVYGFHPRAVHVKWMKNG-DDVPDYETTHTLPNPDGTYQIRVSAEVIPKEGESYSCYVDHSSIGEPLNVVWE
Bufbuf Q9-like X3         VQPQVKVSGQKKGDTMMLHCHVYGFHPRAVHVKWMKNG-DDVPDYETTHTLPNPDGTYQIRVSAEVIPKEGESYSCYVDHSSIGEPLNV---
Bufbuf F10-like (b)       VQPQVKVSGQKKGDTMMLHCQVYGFHPRAVHVKWMKNG-DDVPDYETTHTLPNPDGTYQIRVSAEVIPKEGESYSCYVDHSSIGEPLNVVWE
Bufbuf F10-like(a)        VRPAVKVTGRKSDKVTKLHCLVYGFHPRAVDVKWMKNGIDEVPSYETTHVLPNPDGTYQIRVSVEVIPKEGESYSCYVDHSSIKEPLLVKLE
Bufbuf F10-like X1        VQPGVKVQGQESIKVTKLHCHVYGFHPRAVDVKWMKNEVDDIPSYETTHVLPNPDGTYQIRVSVEVIPKEGESYSCYVDHSSIKEPLLVKWE

Xenlae MHC I              VHPHVRISDHQSADATEIRCQAYGFYPREIDVKWVKNGGDDVHSEAAKEILPNPDGSYQLRVTAEITPNEGDSYACHVEHSSINEKLIVVWP
Xentro MHC I              VRPHVKISDHQSDDITEIRCQAYGFYPREIDVKWVRNGRDDVHSDEAKEILPNPDGSYQLRVTAKVTPKEGDSYACHVDHSSIEEKLIVVWP
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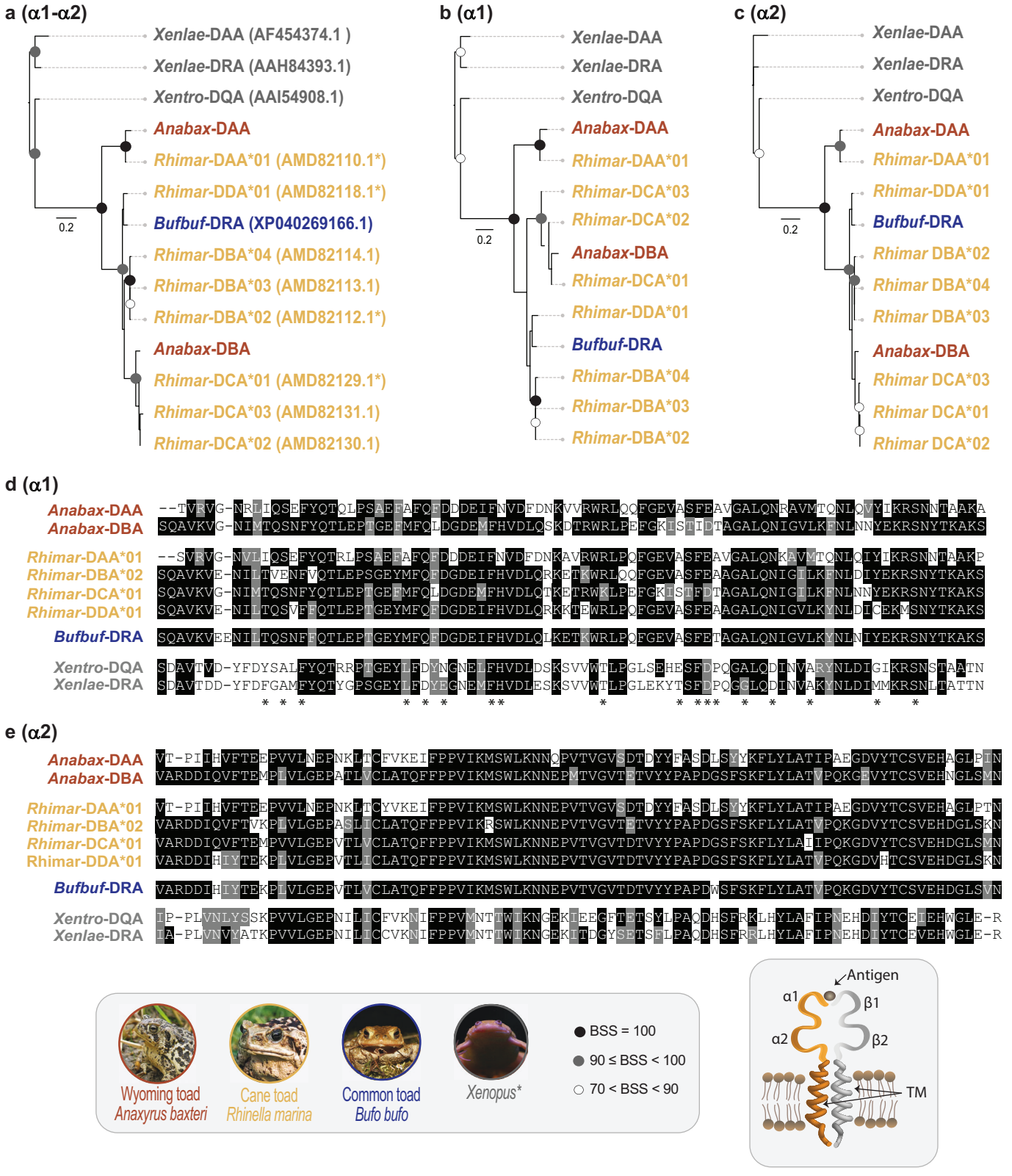
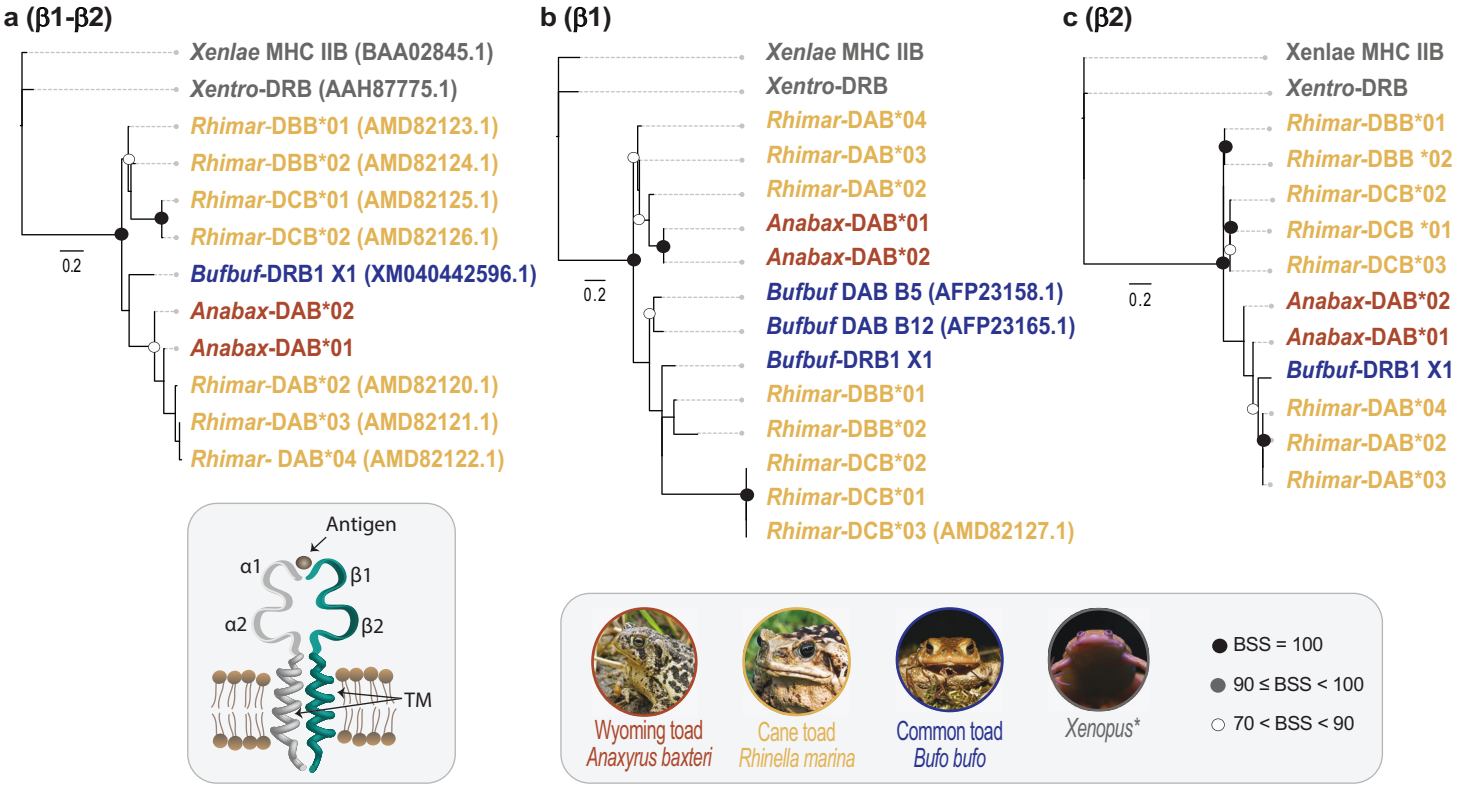

a (α1-α2)
Xenlae-DAA (AF454374.1)
Xenlae-DRA (AAH84393.1)
Xentro-DQA (AAI54908.1)
Anabax-DAA
Rhimar-DAA*01 (AMD82110.1*)
Rhimar-DDA*01 (AMD82118.1*)
Bufbuf-DRA (XP040269166.1)
Rhimar-DBA*04 (AMD82114.1)
Rhimar-DBA*03 (AMD82113.1)
Rhimar-DBA*02 (AMD82112.1*)
Anabax-DBA
Rhimar-DCA*01 (AMD82129.1*)
Rhimar-DCA*03 (AMD82131.1)
Rhimar-DCA*02 (AMD82130.1)
0.2
b (α1)
Xenlae-DAA
Xenlae-DRA
Xentro-DQA
Anabax-DAA
Rhimar-DAA*01
Rhimar-DCA*03
Rhimar-DCA*02
Anabax-DBA
Rhimar-DCA*01
Rhimar-DDA*01
Bufbuf-DRA
Rhimar-DBA*04
Rhimar-DBA*03
Rhimar-DBA*02
0.2
c (α2)
Xenlae-DAA
Xenlae-DRA
Xentro-DQA
Anabax-DAA
Rhimar-DAA*01
Rhimar-DDA*01
Bufbuf-DRA
Rhimar DBA*02
Rhimar DBA*04
Rhimar DBA*03
Anabax-DBA
Rhimar DCA*03
Rhimar DCA*01
Rhimar DCA*02
0.2
d (α1)
Anabax-DAA --TVRVG-NRLIQSEFYQTQLPSAEFAFQFDDDEIFNVDFDNKVVRWRLQQFGEVASFEAVGALQNRAVMTQNLQVYIKRSNNTAAKA
Anabax-DBA SQAVKVG-NIMTQSNFYQTLEPTGEFMFQLDGDEMFHVDLQSKDTRWRLPEFGKISTIDTAGALQNIGVLKFNLNNYEKRSNYTKAKS
Rhimar-DAA*01 --SVRVG-NVLIQSEFYQTRLPSAEFAFQFDDDEIFNVDFDNKAVRWRLPQFGEVASFEAVGALQNKAVMTQNLQIYIKRSNNTAAKP
Rhimar-DBA*02 SQAVKVE-NILTVENFVQTLEPSGEYMFQFDGDEIFHVDLQRKETKWRLQQFGEVASFEAAGALQNIGILKFNLDIYEKRSNYTKAKS
Rhimar-DCA*01 SQAVKVG-NIMTQSNFYQTLEPTGEFMFQLDGDEMFHVDLQTKETRWKLPEFGKISTFDTAGALQNIGILKFNLNNYEKRSNYTKAKS
Rhimar-DDA*01 SQAVKVE-NILTQSVFFQTLEPTGEYMFQFDGDEIFHVDLQRKKTEWRLPQFGEVASFEAAGALQNIGVLKYNLDICEKMSNYTKAKS
Bufbuf-DRA SQAVKVEENILTQSNFFQTLEPTGEYMFQFDGDEIFHVDLQLKETKWRLPQFGEVASFETAGALQNIGVLKYNLNIYEKRSNYTKAKS
Xentro-DQA SDAVTVD-YFDYSALFYQTRRPTGEYLFDYNGNELFHVDLDSKSVVWTLPGLSEHESFDPQGALQDINVARYNLDIGIKRSNSTAATN
Xenlae-DRA SDAVTDD-YFDFGAMFYQTYGPSGEYLFDYEGNEMFHVDLESKSVVWTLPGLEKYTSFDPQGGLQDINVAKYNLDIMMKRSNLTATTN
* * * * * * ** * * *** * * * * *
e (α2)
Anabax-DAA VT-PIIHVFTEEPVVLNEPNKLTCFVKEIFPPVIKMSWLKNNQPVTVGVSDTDYYFASDLSYYKFLYLATIPAEGDVYTCSVEHAGLPIN
Anabax-DBA VARDDIQVFTEMPLVLGEPATLVCLATQFFPPVIKMSWLKNNEPMTVGVTETVYYPAPDGSFSKFLYLATVPQKGEVYTCSVEHNGLSMN
Rhimar-DAA*01 VT-PIIHVFTEEPVVLNEPNKLTCYVKEIFPPVIKMSWLKNNEPVTVGVSDTDYYFASDLSYYKFLYLATIPAEGDVYTCSVEHAGLPTN
Rhimar-DBA*02 VARDDIQVFTVKPLVLGEPASLICLATQFFPPVIKRSWLKNNEPVTVGVTETVYYPAPDGSFSKFLYLATVPQKGDVYTCSVEHDGLSKN
Rhimar-DCA*01 VARDDIQVFTEMPVVLGEPVTLVCLATQFFPPVIKMSWLKNNEPVTVGVTDTVYYPAPDGSFSKFLYLAIIPQKGDVYTCSVEHDGLSMN
Rhimar-DDA*01 VARDDIHIYTEKPLVLGEPVTLICLATQFFPPVIKMSWLKNNEPVTVGVTDTVYYPAPDGSFSKFLYLATVPQKGDVHTCSVEHDGLSKN
Bufbuf-DRA VARDDIHIYTEKPLVLGEPVTLVCLATQFFPPVIKMSWLKNNEPVTVGVTDTVYYPAPDWSFSKFLYLATVPQKGDVYTCSVEHDGLSVN
Xentro-DQA IP-PLVNLYSSKPVVLGEPNILICFVKNIFPPVMNTTWIKNGEKIEEGFTETSYLPAQDHSFRKLHYLAFIPNEHDIYTCEIEHWGLE-R
Xenlae-DRA IA-PLVNVYATKPVVLGEPNILICCVKNIFPPVMNTTWIKNGEKITDGYSETSFLPAQDHSFRRLHYLAFIPNEHDIYTCEVEHWGLE-R
Wyoming toad
Anaxyrus baxteri
Cane toad
Rhinella marina
Common toad
Bufo bufo
Xenopus*
BSS = 100
90 ≤ BSS < 100
70 < BSS < 90
Antigen
α1
β1
α2
β2
TM



d (β1)

```
Anabax-DAB*01  SAAVDYMTESKFECHYLNGTQRVRYLHRVFYNQEEIVYFDSNKGYYIPKTEFGKPD-ADYWNKDKDIIEDRKSSVETFCKHNYGVWKAGAI-ERK
Anabax-DAB*02  SAAVDYMTESKFECHYLNGTQRVRYLHRVFYNQEEIVYFDSNKGYYIPKTEFGKPD-ADYWNKDKDIIEDRKSSVETFCKHNYGVWKAGAI-ERK

Rhimar-DAB*02  SAAVEYMTEEKFECHYLNGTQQVRYLHRCFYNQEETVYFDSNKGEYIAKTEFGKPD-ADYWNKNKDIIEQRKSAVERFCKHNYGVWKEGAI-ERK
Rhimar-DAB*03  SAAVDYMTEQKAECHYVNGTQQVRYLDRYFYNQEELVYFDSDKGYFIAKTEFGKPD-ADYWNKNKDIIEQRKSAVERFCKHNYGVWKEGAI-ERK
Rhimar-DAB*04  SAAVDYMTEQKAECHYVNGTQQVRYLDRYFYNQEELVYFDSDKGYFIAKTEFGKPD-ADYWNKNKDIIEDEKSAVETYCKHNYGVWKEGAI-ERK
Rhimar-DCB*01  SDFRDYVLAATSECHYVNGTQQVRFLDRYFYNQEETVYFDSEKGYFIAKTELGKPD-AEQYNKDKDFIEQKKSAVETYCKHNYGVIHSV-TADRR
Rhimar-DCB*03  SDFRDYVLATAKECHYVNGTQQVRFLDRYFYNQEETVYFDSEKGFYIAKTELGKPD-AEQYNKNKDFIEQTKSAVETYCKPNYEVNTNVGITDRR
Rhimar-DCB*02  SDVNDFIEAITSECHYVNGTQQVRYLERYIYNQEVYVYFDSDVRYFVGKDDYGRIN-ADIWNKDKNFIEQRRTAMEIFCRPNYELTSMGISGRK
Rhimar-DBB*01  SDVNDFIEAITSECHYVNGTQQVRYLERYIYNQEVYVYFDSDVRYFVGKDDYGRIN-ADIWNKDKNFIEQRRTAMEIFCRPNYELTSMGISGRK
Rhimar-DBB*02  SDVNDFIEAITSECHYVNGTQQVRYLERYIYNQEVYVYFDSDVRYFVGKDDYGRIN-ADIWNKDKNFIEQRRTAMEIFCRPNYELTSMGISGRK

Bufbuf-DRB1    SDVRDFVAATTSECHFLNGTQQVRFLDRYFYNQEEFVYFDSDKGYYIAKTELGKPD-ADYWNKNKDFIEREKSAVERYCKYNYGVLHSFTA-DRR

Xenlae MHC IIB SPPEDFVYQFKGQCYYRNGTDNVRLLWRHYYNLEETDYFDSDVGLFIAKTELGKPD-ADNWNSQKDFLEQTFAAVDTVCRHNYQLDKPYTI-DRK
Xentro-DRB     SPPEDFVYQYKGQCCFRNGTENVRGLDRYIYNQEEIAYFDSDVGFFIAKTELGKKHFADYWNSQKDLLEQKRAETDTVCRYNYQFDKPDVI-DRK
                     * * *           * *      **                 *   **     *  **  *   *   *    **
```

e (β2)

```
Anabax-DAB*01  VEPEIVVALMPNHEEPSTVHHILQCNVFGFYPSEVEVKWYRNGQEETELVTSSALFQNGDWTYRILVMLETEIQKGDTFTCEVHHSSLKAPHRVDWHP
Anabax-DAB*02  VEPEIVVALMPNHEEPSTVHHILQCNVFGFYPSEVEVKWYRNGQEETELVQSTEPYHNGDWSYQILVMLETEIQKGDTFTCEVHHSSLKAPHRVDWHP

Rhimar-DAB*02  VKPEIVVSLMPNHEEPRTVHHILQCNVFGFYPSEVEVKWYRNGQEETELVTSSALFQNGDWSFRILVMLETEIHKGDTFTCEVHHISLEAPHRVDWHP
Rhimar-DAB*03  VKPEIVVSLMPNHEEPRTVHHILQCNVFGFYPSEVEVKWYRNGQEETELVTSSALFQNGDWSFRILVMLETEIHKGDTFTCEVHHISLEAPHRVDWHP
Rhimar-DAB*04  VKPEIVVSLMPNHEEPRTVHHILQCNVFGFYPSEVEVKWYRNGQEETELVTSSALFQNGDWSFRILVMLETEIHKGDTFTCEVHHISLEAPHRVDWHP
Rhimar-DCB*01  VEPSIKISLMNQYEESHTKQHMLICNVFGFYPSVIEVKWYRNGQEETELVQSTEPYHNGDWSYQILVMLETEIQKGDTFTCEVHHSSLKDPHRVDWHP
Rhimar-DCB*03  VEPSIKISLMNQYEESHTKQHMLICNVFGFYPSVIEVKWYRNGQEETELVQSTEPYHNGDWSYQILVMLETEIQKGDTFTCEVHHSSLKDPHRVDWHP
Rhimar-DCB*02  VQPSIKISLMNQYEDSYSKQHMLICNVFGFYPSEIEVKWYRNGQEETELVQSTTPYHNGDWSFQILVMLETEIQKGDTFTCEVHHSSLKDPHRVDWYP
Rhimar-DBB*01  VQPSIKISLMNQYEDSYSKQHMLICNVFGFYPSEIEVKWYRNGQEETELVQSTTPYHNGDWSFQILVMLETEIQKGDTFTCEVHHSSLKDPHRVDWYP
Rhimar-DBB*02  VQPSIKISLMNQYEDSYSKQHMLICNVFGFYPSEIEVKWYRNGQEETELVQSTTPYHNGDWSFQILVMMETEIQKGDTFTCEVHHSSLKDPHRVDWYP

Bufbuf-DRB1    VQPEIVVSLLPRHEEPSTVHHLLQCNVFGFYPSVVEVKWYRNSQEETELVTSSVLFQNGDWTFQILVMLETEIHSSDTFTCEVHHSSLEAPHRVDWHP

Xenlae MHC IIB SQPNVKIVNT--KTLDLEHENLITCFVDGFFPPMIKVTWLKNGIEEGEQVTSSELLQNGDWTFEIHVMLETTIKHGDTFTCRVEHSSLQQFVSVNWEP
Xentro-DRB     SQPNVKIVNS--KTLDLEHENLITCFVDGFFPPMIKVTWLKNGIEEGEQVTSSELLPNGDWTFEIHVMLETTIKHGDTFTCRVEHSSLQQFISVNWEP
```

Supplementary Tables and Figures

Conservation Genetics

Transcriptome annotation reveals minimal immunogenetic diversity among Wyoming toads, *Anaxyrus baxteri*

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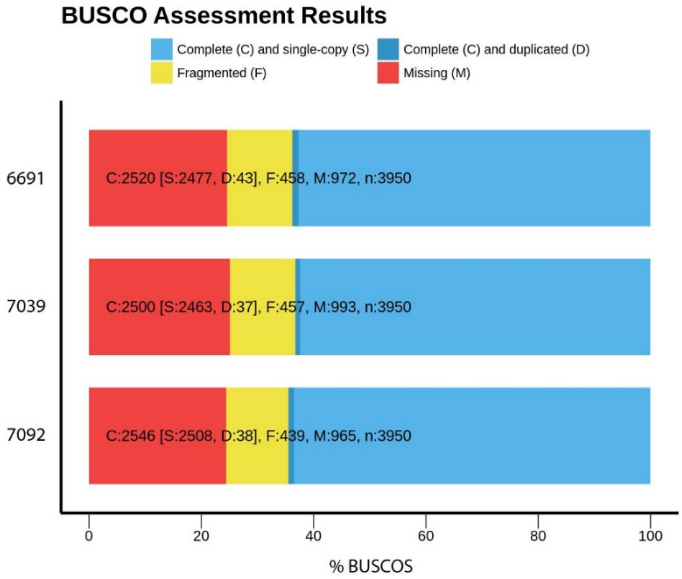
Contents:

Supplementary Table S1. Summary of Wyoming toad transcriptome analysis

	Toad 6691	Toad 7039	Toad 7092
Total Trinity Genes	245,790	134,581	143,205
Total Trinity Transcripts	332,088	199,248	209,600
Percent GC	45.07	45.31	45.41
Contig N50 (ALL)	1,709	2,041	2,038
Median Contig Length (ALL)	371	426	425
Avg Contig (ALL)	820.4	969.95	961.91
Total Assembled Bases (ALL)	272,445,109	193,259,855	201,616,519
Contig N50 (LONGEST)	709	1,096	1,044
Median Contig Length (LONGEST)	319	337	337
Avg Contig (LONGEST)	555.6	660.94	652.23
Total Assembled Bases (LONGEST)	136,560,916	88,949,732	93,402,820
Total Paired Reads	79,216,573	61,479,313	65,858,371
Overall Alignment Rate	91.63%	90.71%	91.31%

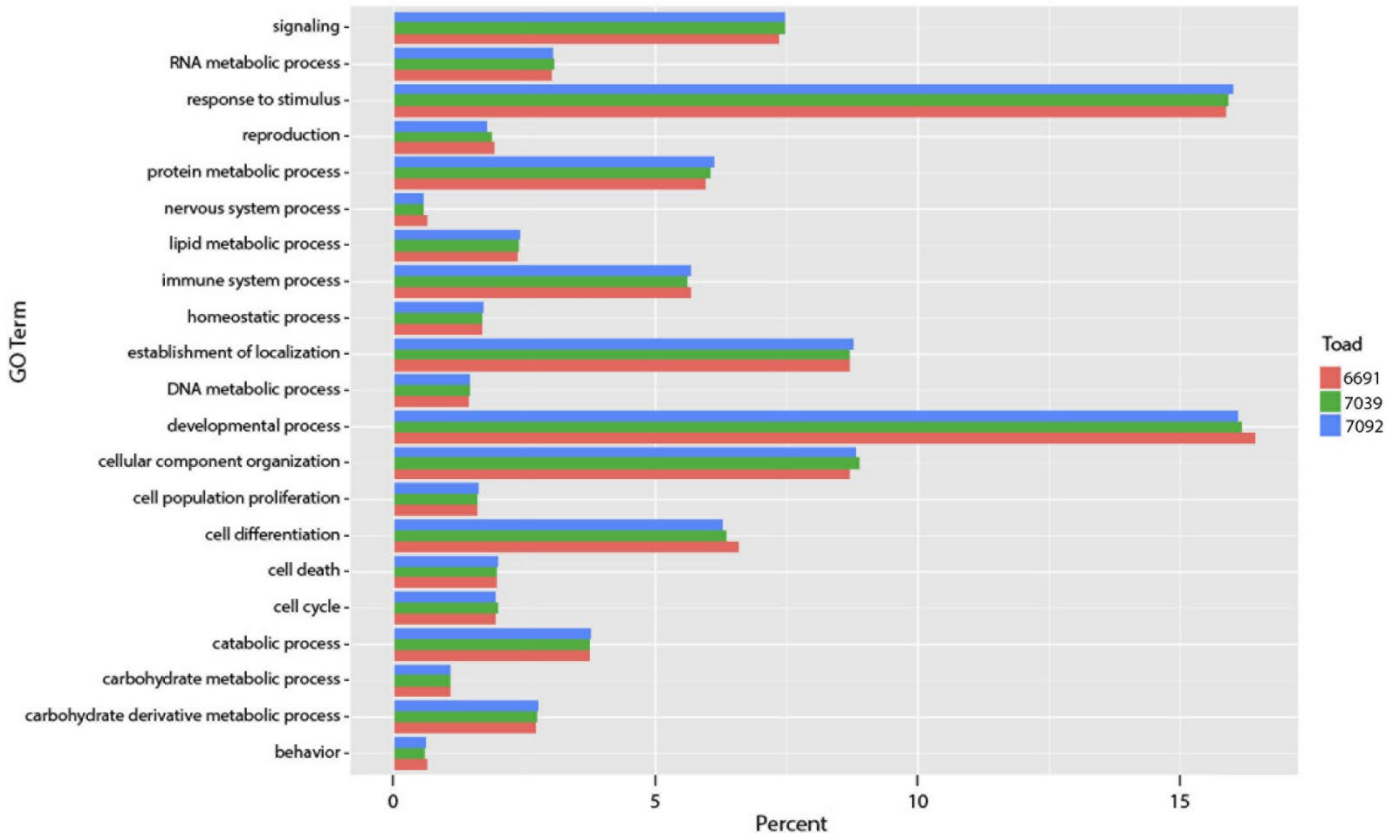


Supplementary Fig. S1. BUSCO assessment results from Wyoming toad transcriptomes. BUSCO tetrapod orthologs were cataloged as complete and single copy, complete and duplicated, fragmented or missing in each of the three toads (6691, 7039 and 7092).

Supplementary Table S2. Duplicated sequences in all three Wyoming toads*

BUSCO ID	OrthoDB Annotation	Human Uniprot ID
EOG090701VI	thyroid peroxidase	P07202
EOG0907020N	DEAH box polypeptide	Q8IX18
EOG090702QL	HBS1-like translational GTPase	D9YZV0
EOG0907036F	peroxisomal biogenesis factor 6	A0A024RD09
EOG090703A7	transketolase	P29401
EOG090704A2	flavin containing monooxygenase 4	P31512
EOG090705SC	cholinergic receptor	P02708
EOG090705ZM	tubby like protein 3	O75386
EOG090706G4	dopamine receptor	P14416
EOG090706LB	serine/threonine kinase	O94768
EOG0907071S	Nucleobindin 2	V9HW75
EOG090708E2	Nephrosis 2	Q9NP85
EOG090708FT	NIPA-like domain containing 4	Q0D2K0
EOG090709BR	potassium channel	Q96T55
EOG09070A0P	Coiled-coil domain containing 3	Q9BQI4

* BUSCO analysis, which identifies ultra-conserved, single copy genes, revealed fifteen duplicate genes in all three Wyoming toads. These genes have been annotated via OrthoDB, however further enrichment analysis is impossible with such a small set of genes.



Supplementary Fig. S2. Summary of annotated transcriptomes based on GO terms
Gene ontology (GO) terms from Wyoming toad annotated transcriptomes and each group's percent contribution to total transcriptome. Biological process GO terms were chosen for this analysis as this category includes the terms immune system processes and response to stimulus which include important immune gene annotations. All three toads displayed comparable proportions of GO terms to one another..

Supplementary Table S3. Representative Wyoming toad TLR transcripts and GenBank IDs

Sequence name	Toad 6691	Toad 7039	Toad 7092
TLR1a	GGUS01160622.1	GGUR01153850.1	GGUQ01094842.1
TLR1b	GGUS01148135.1	GGUR01053790.1	GGUQ01193029.1
TLR2	GGUS01236735.1	Not identified	GGUQ01144158.1
TLR3	GGUS01239741.1	GGUR01125592.1	GGUQ01122259.1
TLR4	GGUS01036104.1	GGUR01092862.1	GGUQ01142735.1
TLR5	GGUS01267723.1	GGUR01011083.1	GGUQ01091435.1
TLR8a	GGUS01251716.1	Not identified	GGUQ01056653.1
TLR8b	Not identified	GGUR01052356.1	Not identified
TLR12	GGUS01208850.1	GGUR01100539.1	GGUQ01123333.1
TLR13	GGUS01005708.1	GGUR01162624.1	GGUQ01081784.1
TLR14	GGUS01235967.1	GGUR01048765.1	GGUQ01096496.1
TLR21	GGUS01313948.1	Not identified	GGUQ01185422.1

Supplementary Table S4. Representative Wyoming toad MHC class I transcripts and GenBank IDs

Sequence name	Toad 6691	Toad 7039	Toad 7092
*Anabax-UA*01*	Not identified	GGUR01113130.1	Not identified
*Anabax-UA*02*	GGUS01278791.1	Not identified	GGUQ01004097.1
*Anabax-UB*01*	Not identified	GGUR01024835.1	GGUQ01038068.1
*Anabax-UB*02*	GGUS01018260.1	Not identified	Not identified
*Anabax-UC*01*	GGUS01183637.1	Not identified	GGUQ01038171.1
*Anabax-UC*02*	Not identified	GGUR01080688.1	GGUQ01038022.1
*Anabax-UD*01*	GGUS01214789.1	GGUR01025091.1	Not identified
*Anabax-UD*02*	Not identified	Not identified	GGUQ01038222.1
*Anabax-UD*03*	GGUS01191828.1	Not identified	Not identified
β-2-microgobulin	GGUS01148031.1	GGUR01189603.1	GGUQ01034849.1

```
                    SP
Anabax-UA*01     1  MFPLIFLLLYVSAVYSDTHSLRYYMTGVSAPGSGLPEYSEVGYVDDREIVNYNSESGRME
Anabax-UA*02     1  MFPLIFLLLYVSAVYSDTHSLRYYMTGVSAPGSGLPEYSEVGYVDDREIVNYNSESGRME

Anabax-UA*01    61  PKVKWMEKVDPGYWERNTQIAKGNEAVNKHNVRTLMSRFNQTGGFHIVQAMYGCERRDDG
Anabax-UA*02    61  PKVKWMEKVDPGYWERNTQIAKGNEAVNKHNVRTLMSRFNQTGGFHIVQWMYGCERIDDG

Anabax-UA*01   121  GITVYDQHGYDGGEFMSLDTQTWTEIPTMSQAQITAQRWNSPEEQWGQRYKNYLEIECKD
Anabax-UA*02   121  GITGYDQHGYDGGEFMSLDTQTWTYIPTMSQAQITAQRWNSPEEQWGQRYKNYLEIECKD

Anabax-UA*01   181  WLQKYVENGREDLERRVQPQVKVSGQKKDDAMMLHCQVYGFHPRPVHVKWMKNKDDVHSY
Anabax-UA*02   181  WLQKYVENGREDLERRVQPQVKVSGQKKDDAMMLHCQVYGFHPRPVHVKWMKNKDDVHSY

                                                                        TM
Anabax-UA*01   241  ETTHTLPNPDGTYQIRVSAEVIPKEGESYSCYVDHSSLKEPLNIVWEPSNRTVWVTPVVI
Anabax-UA*02   241  ETTHTLPNPDGTYQIRVSAEVIPKEGESYSCYVDHSSLKEPLNIVWEPSNRTVWVTPVVI

                    TM
Anabax-UA*01   301  AVVVILLAVLGIGGFLLYRRKKPDYKATSTSDTSSSDASDNAAKA*
Anabax-UA*02   301  AVVVILLAVLGIGGFLLYRRKKPDYKATSTSDTSSSDASDNAAKA*
```

Supplementary Fig. S3. Wyoming toad MHC class I UA sequences.
The two major forms of *Anabax-UA* were aligned. α1, α2, and α3 domains are color-coded blue, red and yellow, respectively. Signal peptide (SP) and transmembrane (TM) domains are indicated above the alignment. Identical residues are shaded the same color, except for gray which reflects structurally similar residues. An asterisk indicates the presence of a stop codon.

```
                    SP
Anabax-UB*01     1  MEMYALTLILLCTSGAYSDSHSLYYCYTGVTAPGSGLPEFSIVGYMDDQQTELYNSDIGK
Anabax-UB*02     1  -------------------SHSLRYYYTGVSAPGSGLPEFSIVGYMDDQQTELYNSDIGK

Anabax-UB*01    61  CIPVATWVRKERPEQWLKKTLTSKANEALFKHEVKIVMKRFNHTEGLHFAQVMHSCELKD
Anabax-UB*02    42  CIPVATWVRKERPEQWLKKTLTSKANEALFKHEVKIVMKRFNHTEGLHFAQVMHSCELKD

Anabax-UB*01   121  DGSIVSYEEFRYDGREYMYLDIKTGLFIPTMAEAQITTQRWNSPDVRAGQRIRNYLANEC
Anabax-UB*02   102  DGSIVSYEEFRYDGREYMYLDIKTGLFIPTMAEAQITTQRWNSPDVRAGQRIRNYLANEC

Anabax-UB*01   181  IDRLRRYVVYGREDLERRVQPGVKVTGRESGEITKLHCLVYGFHPRAVDVKWMKNGIDEI
Anabax-UB*02   162  IDRLRRYVVYGREDLERRVQPGVKVTGRESGEITKLHCLVYGFHPRAVDVKWMKNGIDEI

Anabax-UB*01   241  PSYETTHVLPNPDGTYQIRVSVEVIPKEGESYSCY
Anabax-UB*02   222  PSYETTHVLPNPDGTYQIRVSVEVIPKEGESYS--
```

Supplementary Fig. S4. Wyoming toad MHC class I UB sequences.
The two major forms of *Anabax-UB* were aligned. α1, α2, and α3 domains are color-coded blue, red and yellow, respectively. Signal peptide (SP) domains are indicated above the alignment. Identical residues are shaded the same color, except for gray which reflects structurally similar residues. Neither of these transcripts encode a stop codon and are likely 3' truncated.

```
                        SP
Anabax-UC*01     1  MMEKMCSLALVLLSLSGAYSDSHSLRYYSIGVSAPGSGLPEFSIIGYVDDQQIELYSGDT
Anabax-UC*02     1  MMEKMCSLALVLLSLSGAYSDSHSLRYYSIGVSAPGSGLPEFSIIGYVDDQQIELYSGDT

Anabax-UC*01    61  GRSVPVAPWLSRNVGLEHWERRTRISKEYEALFKHEVKAAVKRFNHTGGFHFVQVMHNCE
Anabax-UC*02    61  GRSVPVAPWLSRNVGLEHWERRTRISKEYEALFKHEVKAAVKRFNHTGGFHFVQVMHNCE

Anabax-UC*01   121  MRDDGSTTGHQEYRYDGEEYMYLDIKSALFNPTMAEAQIITQRWNSPDIRKGEREKNYLE
Anabax-UC*02   121  MRDDGSTTGHQEYRYDGEEYMYLDIKSALFNPTMAEAQIITQRWNSPDIRKGEREKNYLE

Anabax-UC*01   181  SKCIERLKKYLEFGREDLERRGDFGLSHVYNFATVPH*
Anabax-UC*02   181  SKCIERLKKYLEFGREDLERRVQPGVKVTG-------
```

Supplementary Fig. S5. Wyoming toad MHC class I UC sequences.
The two major forms of *Anabax-UC* were aligned. α1, and α2 domains are color-coded blue and red, respectively. Signal peptide (SP) domains are indicated above the alignment. Identical residues are shaded the same color, except for gray which reflects structurally similar residues. An asterisk indicates the presence of a stop codon.

```
                        SP
Anabax-UD*01     1  MEMYALTLILLCTSGAYSDSHSLYYCYTGVTAPGSGLPEFSVVGYMDGQQVELYTSDIGR
Anabax-UD*02     1  MEMYALTLILLCTSGAYSDSHSLYYCYTGVTAPGSGLPEFSVVGYMDGQQVELYTSDIGR
Anabax-UD*03     1  MEMYALTLILLCTSGAYSDSHSLYYCYTGVTAPGSGLPEFSVVGYMDGQQVELYTSDIGR

Anabax-UD*01    61  SVPVAHWLKEKEDSKFWDELTRIRQYSETFFRNELKIAVKRFNHTKGFHYVQAMLGCELR
Anabax-UD*02    61  SVPVAHWLKEKEDSKFWDELTRIRQYSETFFRNELKIAVKRFNHTKGFHYVQAMLGCELR
Anabax-UD*03    61  SVPVAHWLKEKEDSKFWDELTRIRQYSETFFRNELKIAVKRFNHTKGFHYVQAMLGCELR

Anabax-UD*01   121  DDGSTIGFNQYANDGSEFLFLDLQTKTFIPTMAEAQIITQKWNSWESGIRERVKDNDIVC
Anabax-UD*02   121  DDGSTIGFNQYANDGSEFLFLDLQTKTFIPTMAEAQIITQKWNSWESGIRERVKDNDIVC
Anabax-UD*03   121  DDGSTIGFNQYANDGSEFLFLDLQTKTFIPTMAEAQIITQKWNSWESGIRERVKDNDIVC

Anabax-UD*01   181  INRLNRYLKHGRQHLERRVQPGVKVTGRESGEVTKLHCHVYGFHPRAVDVKWMKNGIDEI
Anabax-UD*02   181  INRLNRYLKHGRQHLERRVQPGVKVTGRESGEVTKLHCHVYGFHPRAVDVKWMKNE----
Anabax-UD*03   181  INRLNRYLKHGRQHLERRGDTAHYLYQVFLL------VIMYSR-ENKYLMHWRFCKFSHL

Anabax-UD*01   241  PSYETTHVLPNPDGTYQIRVSVEVIPKEGESYS
Anabax-UD*02        --------------------------------
Anabax-UD*03   234  QRMEMSVIFI-----------------IGR---
```

Supplementary Fig. S6. Wyoming toad MHC class I UD sequences.
The three major forms of *Anabax-UC* were aligned. α1, α2, and α3 domains are color-coded blue, red and yellow, respectively. Signal peptide (SP) domains are indicated above the alignment. Identical residues are shaded the same color, except for gray which reflects structurally similar residues. None of these transcripts encode a stop codon and are likely 3' truncated.

Supplementary Table S5. Representative Wyoming toad MHC class II transcripts and GenBank IDs

Sequence name	Toad 6691	Toad 7039	Toad 7092
Anabax-DAA	GGUS01080349.1	GGUR01117947.1	GGUQ01148610.1
Anabax-DBA	GGUS01104323.1	GGUR01016231.1	GGUQ01047262.1
*Anabax-DAB*01*	GGUS01279675.1	GGUR01053143.1	GGUQ01049761.1
*Anabax-DAB*02*	Not identified	GGUR01053272.1	GGUQ01049863.1
Truncated novel class II β1 domain	GGUS01258414.1	GGUR01110179.1	GGUQ01029310.1
Truncated novel class II β1 domain	GGUS01090892.1	GGUR01054024.1	GGUQ01039448.1

```
                    SP
Anabax-DAA      1 MIGRKHMYLALICALCLKVIQTVRV---GNRLIQSEFYQTQLPSAEFAFQFDDDEIFNVD
Anabax-DBA      1 MSPRKLLNLGLVCWMCIFYVHTSQAVKVGNIMTQSNFYQTLEPTGEFMFQLDGDEMFHVD

Anabax-DAA     58 FDNKVVRWRLQQFGEVASFEAVGALQNRAVMTQNLQVYIKRSNNTAA-KAVTPIIHVFTE
Anabax-DBA     61 LQSKDTRWRLPEFGKISTIDTAGALQNIGVLKFNLNNYEKRSNYTKAKSVARDDIQVFTE

Anabax-DAA    117 EPVVLNEPNKLTCFVKEIFPPVIKMSWLKNNQPVTVGVSDTDYYFASDLSYYKFLYLATI
Anabax-DBA    121 MPLVLGEPATLVCLATQFFPPVIKMSWLKNNEPMTVGVTETVYYPAPDGSFSKFLYLATV

                                                          TM
Anabax-DAA    177 PAEGDVYTCSVEHAGLPINPTNKFWIPEAPSHLSETSENVVCGLGLAVGIIGIVAGIFLI
Anabax-DBA    181 PQKGEVYTCSVEHNGLSMNPTNKFWLPELPQPISETSDNILCGIGLAYGIFGIVAGIVLI

Anabax-DAA    237 FKGLKNNNQNRGH*
Anabax-DBA    241 CKGKKNSNQAGGH*
```

Supplementary Fig. S7. Wyoming toad MHC class II alpha chain sequences.
Wyoming toad DAA and DBA were aligned. Identical residues within the α1 and α2 domains are color-coded blue and red, respectively. Signal peptide (SP) and transmembrane (TM) domains are indicated above the alignment. Identical residues are shaded the same color, except for gray which reflects structurally similar residues. An asterisk indicates the presence of a stop codon.

```
                    SP
Anabax-DAB*01     1 MGMLYCISFIFFILLIRLDFCRLSAAVDYMTESKFECHYLNGTQRVRYLHRVFYNQEEIV
Anabax-DAB*02     1 MGMLYCISFIFFILLIRLDFCRLSAAVDYMTESKFECHYLNGTQRVRYLHRVFYNQEEIV

Anabax-DAB*01    61 YFDSNKGYYIPKTEFGKPDADYWNKDKDLIEDRKSSVETFCKHNYGVWKAGAIERKVEPE
Anabax-DAB*02    61 YFDSNKGYYIPKTEFGKPDADYWNKDKDLIEDRKSSVETFCKHNYGVWKAGAIERKVEPE

Anabax-DAB*01   121 IVVALMPNHEEPSTVHHILQCNVFGFYPSEVEVKWYRNGQEETELVTSSALEQNGDWTYR
Anabax-DAB*02   121 IVVALMPNHEEPSTVHHILQCNVFGFYPSEVEVKWYRNGQEETELVQSTEPYHNGDWSYQ

                                                          TM
Anabax-DAB*01   181 ILVMLETEIQKGDTFTCEVHHSSLKAPHRVDWHPQMSESAKSKRATGIGGIVLGTAFLIV
Anabax-DAB*02   181 ILVMLETEIQKGDTFTCEVHHSSLKAPHRVDWHPQSSDAAKNKMATGIVGFVLGVVFFIV

                    TM
Anabax-DAB*01   241 GIVIYMRGRKGQTPFRVPQSDHFPHT*
Anabax-DAB*02   241 GLVIYMRARKGQMSFPGQQSERFLPQ*
```

Supplementary Fig. S8. Wyoming toad MHC class II beta chain sequences.
Wyoming toad DAB*01 and DAB*02 were aligned. Identical residues within the β1 and β2 domains are color-coded blue and red, respectively. Signal peptide (SP) and transmembrane (TM) domains are indicated above the alignment. Identical residues are shaded the same color, except for gray which reflects structurally similar residues. An asterisk indicates the presence of a stop codon.

Supplementary Table S6. Sequence identity between bufonid TLR ectodomains*

	TLR8a, CMT	TLR8a, CNT	TLR8b, WYT	TLR8a, WYT	TLR8b, CMT	TLR8b, CNT
TLR8a, CMT		92.87	52.26	52.26	52.73	52.26
TLR8a, CNT	92.87		52.24	52.24	53.44	52.34
TLR8b, WYT	52.26	52.24		100	88.8	89.49
TLR8a, WYT	52.26	52.24	100		88.8	89.49
TLR8b, CMT	52.73	53.44	88.8	88.8		90.21
TLR8b, CNT	52.26	52.34	89.49	89.49	90.21	

* GenBank sequence identifiers for Wyoming toad (WYT) TLRs are in **Supplemental Table S3** and sequence identifiers for common toad (CMT) and cane toad (CNT) TLRs are included in **Figure 2**.

Supplementary Table S7. Sequence identity between bufonid TLR TIR domains*

	TLR8a, WYT	TLR8a, CMT	TLR8a, CNT	TLR8b, CNT	TLR8b, WYT	TLR8b, CMT
TLR8a, WYT		96.05	97.37	87.5	88.82	88.16
TLR8a, CMT	96.05		98.68	87.5	87.5	86.84
TLR8a, CNT	97.37	98.68		88.82	88.82	88.16
TLR8b, CNT	87.5	87.5	88.82		97.37	96.71
TLR8b, WYT	88.82	87.5	88.82	97.37		97.37
TLR8b, CMT	88.16	86.84	88.16	96.71	97.37	

* GenBank sequence identifiers for Wyoming toad (WYT) TLRs are in **Supplemental Table S3** and sequence identifiers for common toad (CMT) and cane toad (CNT) TLRs are included in **Figure 2**.