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Selection, trans-species polymorphism, and locus identification of major histocompatibility complex class IIβ alleles of New World ranid frogs

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Abstract Genes encoded by the major histocompatibility complex (MHC) play key roles in the vertebrate immune system. However, our understanding of the evolutionary processes and underlying genetic mechanisms shaping these genes is limited in many taxa, including amphibians, a group currently impacted by emerging infectious diseases. To further elucidate the evolution of the MHC in frogs (anurans) and develop tools for population genetics, we surveyed allelic diversity of the MHC class II β1 domain in both genomic and complementary DNA of seven New World species in the genus *Rana* (*Lithobates*). To assign locus affiliation to our alleles, we used a "gene walking" technique to obtain intron 2 sequences that flanked MHC class IIβ exon 2. Two distinct intron sequences were recovered, suggesting the presence of at least two class IIβ loci in *Rana*. We designed a primer pair that successfully amplified an orthologous locus from all seven *Rana* species. In total, we recovered 13 alleles and documented trans-species polymorphism for four of the alleles. We also found quantitative evidence of selection acting on amino acid residues that are putatively involved in peptide binding and structural stability of the β1 domain of anurans. Our results indicated that primer mismatch can result in polymerase chain reaction (PCR) bias, which influences the number of alleles that are recovered. Using a single locus may minimize PCR bias caused by primer mismatch, and the gene walking technique was an effective approach for generating single-copy orthologous markers necessary for future studies of MHC allelic variation in natural amphibian populations.

Keywords Amphibia · Beta chain · Gene walking · *Lithobates* · Positive selection · *Rana*

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Introduction

Major histocompatibility complex (MHC) genes are central regulators of antigen-specific immune responses across vertebrates, making them common targets for immunogenetic disease association studies in non-model systems (reviewed in Bernatchez and Landry 2003). Antigen-presenting MHC molecules are encoded by two structurally and functionally distinct gene families designated class I and class II (Klein and Horejsi 1997; Marsh et al. 2000). Traditional views of MHC function dichotomize these loci, with class I MHC molecules presenting intracellular pathogen peptides to $CD8^+$ T lymphocytes and class II MHC molecules presenting peptides from extracellular pathogens to $CD4^+$ T lymphocytes (Bevan 1987). However, substantial crosstalk occurs during acquired immune responses, and cross-presentation of extracellular peptides by class I and intracellular peptides by class II can also occur (Vyas et al. 2008). Single MHC molecules can determine an organism's response to particular pathogens, which can put MHC loci under intense balancing selection

promoting long persistence of allelic lineages (Takahata and Nei 1990) and the presence of similar or identical alleles in multiple species (trans-species polymorphism: Klein 1987; Takahata 1990).

Despite the high number of MHC disease association studies in non-model species (e.g., Hedrick et al. 2001; Langefors et al. 2001; Arkush et al. 2002; Grimholt et al. 2003), characterization of locus-specific alleles in non-model organisms is rare. This discrepancy exists because most studies exploit the extensive similarity in particular regions of MHC coding sequences to amplify MHC genes using degenerate polymerase chain reaction (PCR) procedures. While this is an effective tool for amplifying uncharacterized MHC loci, an issue with this approach is the simultaneous amplification of exons from all MHC genes, rendering orthologous loci indistinguishable from paralogous loci (e.g., Hauswaldt et al. 2007).

One way to determine locus identity for recovered alleles is to observe the patterns of adjacent intron sequences. For example, in primate MHC class I loci, the introns adjacent to paralogous exons have diverged to the point of conferring a unique identity on each paralog in the genome (Cereb et al. 1997). While the exons experienced balancing selection that maintained polymorphism, introns were predominately shaped by genetic drift and recombination that homogenized introns within loci. If introns are, as a rule, identifiably unique among paralogs but alignable across species within paralogs, the associated exons can be assigned to specific orthologous loci, which are more suitable for comparative analyses. In the present study, we implement a comparative "gene walking" approach to amplify exon and adjacent intron sequences. We investigated locus identity of MHC class IIβ alleles in multiple species and developed locus-specific markers, with the ultimate objective of providing infrastructure for future comparative population genetic analyses. Similar approaches, such as vectorette PCR, have been used to successfully amplify introns from a single species (e.g., Babik et al. 2008, Ko et al. 2003).

MHC loci have been identified in all major vertebrate lineages, but the complex remains understudied in most amphibians, aside from the closely related model organisms *Xenopus laevis* and *Silurana* (*Xenopus*) *tropicalis*. As in other vertebrates, the products of the anuran MHC class II genes form αβ heterodimers which function in antigen presentation (Flajnik and Du Pasquier 1990) and demonstrate molecular signatures of positive Darwinian selection (Du Pasquier et al. 1989; Sato et al. 1993). Developmental (Du Pasquier and Flajnik 1990), genetic (Sato et al. 1993; Nonaka et al. 1997), and functional assays (Du Pasquier et al. 1989; Flajnik and Du Pasquier 1990) show that *Xenopus* has at least two class IIα and two class IIβ loci (Sato et al. 1993; Ohta et al. 2006). Recent molecular characterization of MHC class II in the fire-bellied toad (*Bombina bombina*) revealed similar structure and function of class IIβ alleles, with a minimum of two class IIβ loci expressed (Hauswaldt et al. 2007). *Bombina*, *Xenopus*, and *Silurana* are members of lineages that diverged early in the anuran tree (Frost et al. 2006); the majority of extant frog species fall within the Neobatrachia, a clade that possesses numerous derived features. To date, the characterization of MHC alleles has only been accomplished in two Neobatrachian species: the common frog, *Rana* (*Lithobates*) *temporaria* (class I: Teacher et al. 2009; class II: Hauswaldt et al. 2007; Zeisset and Beebee 2009), and the natterjack toad, *Bufo calamita* (class II: May and Beebee 2009). A growing interest in amphibian immunogenetics has been precipitated by the recent emergence of several amphibian infectious diseases (e.g., Berger et al. 1998; Gray et al. 2009). Immunogenetic variability may be an important factor in determining how amphibian populations respond to novel pathogens, and both innate and acquired immune genes may influence whether a particular organism is susceptible (Richmond et al. 2009).

We isolated the β1 domain of MHC class II loci across seven ranid species in the genus *Rana* (*Lithobates*): *Rana catesbeiana*, *Rana clamitans*, *Rana palustris*, *Rana pipiens*, *Rana sylvatica, Rana warszewitschii*, and *Rana yavapaiensis*. We focus on a single New World genus, *Rana*, because different species within this genus show a range of response to novel pathogens; for example, some frogs can be asymptomatic carriers of the fungal pathogen *Batrachochytrium dendrobatidis* (*R. catesbeiana*: Daszak et al. 2004; Garner et al. 2006; *R. pipiens*: Carey et al. 1999), while others are susceptible to the epidermal disease it causes (*R. warszewitschii*: Lips et al. 2006; *R. yavapaiensis*: Bradley et al. 2002). The seven species share close phylogenetic relatedness and are therefore likely to possess orthologous MHC loci and shared allelic lineages. Our goals were to identify individual class IIβ loci, quantify levels of MHC class II polymorphism and selection, and generate markers for use within *Rana* species that might be exposed to emerging diseases.

Materials and methods

Tissue collection and nucleic acid extraction

We extracted genomic DNA from either fresh liver tissue or toe clips (preserved in ethanol) from a single representative of seven ranid species (*R. catesbeiana*, *R. clamitans*, *R. pipiens*, *R. palustris*, *R. yavapaiensis, R. sylvatica*, and *R. warszewitschii*; Electronic supplementary material (ESM) Online Resource 1) and *X. laevis* using Qiagen DNeasy kits (Qiagen, Valencia, CA, USA). Total RNA was extracted

from either spleen or small intestine tissue of six of the seven species (we were unable to obtain RNA from *R. warszewitschii*) using an Agencourt® RNAdvance™ tissue Kit (Beckman Coulter Genomics, Danvers, MA, USA). After a DNase treatment, we generated first-strand complementary DNA (cDNA) using the Superscript One-step PCR kit (Invitrogen, Carlsbad, CA, USA) for reverse transcriptase PCR (50°C for 1 h using an Oligo-DT_{20} primer).

Isolation of MHC class IIβ exon 2 from cDNA and gDNA

First, we broadly screened for total allelic diversity regardless of locus by amplifying a fragment of exon 2 by PCR using published degenerate primer sequences that annealed within exon 2 (MHC-F and MHC-5R; Hauswaldt et al. 2007). We obtained these exon 2 sequences from both genomic DNA (gDNA) and cDNA samples from one individual of each species (except *R. warszewitschii*, for which we only had gDNA). In addition, we amplified alleles from *X. laevis* to compare to those obtained from previous studies. The amplifications were performed for 35 cycles (95°C for 30 s, 52°C for 50 s, 72°C for 1 min) with standard reaction conditions. To verify the exon 2 sequences, a second PCR was run on every individual's cDNA and gDNA a second time, independently, and cloned. Eight clones were sequenced from this second run.

Isolation of MHC class IIβ intron 2

We used a gene walking (or ligation-mediated PCR) technique (described in Cottage et al. 2001) to isolate partial intron 2 sequences from three species, *R. catesbeiana*, *R. clamitans*, and *R. yavapaiensis*, in order to design degenerate primers that could be used to amplify intron 2 sequence from all the *Rana* species used in this study. Briefly, digested gDNA was ligated overnight with T4 ligase to oligonucleotide adaptors to generate adaptor libraries. We performed a first round of selective PCR using the adaptor primer AP1 (Cottage et al. 2001) and a gene-specific primer MHC-F (Hauswaldt et al. 2007). Amplifications were performed for 35 cycles consisting of 94°C for 50 s, 55°C for 45 s, and 72°C for 3 min. PCR products were diluted 1:100 in deionized water, and a second nested PCR was performed using the primers NAP1 (Cottage et al. 2001) and MHC-F01 (Fig. 1) with 30 cycles of 94°C for 50 s, 60°C for 45 s, and 72°C for 2 min.

The gene walking technique provided us with partial intron 2 sequences from *R. yavapaiensis*, *R. clamitans*, and *R. catesbeiana*. These sequences were used to design a ranid intron-specific reverse primer (B1intron2_R; Fig. 1) that amplified a fragment containing both exon 2 and adjacent intron 2 sequences. This primer, paired with MHC-F (Hauswaldt et al. 2007), was used to amplify this region

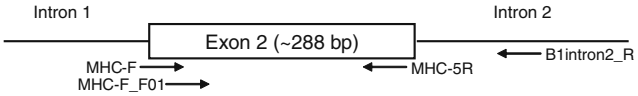

Fig. 1 Illustration of the location of primers used for amplifying a fragment of MHC class IIβ exon 2 and intron 2 from ranid frogs. The B1intron2_R sequence was 5′-CAC ATA ATC CAG TAG TAR AAA GYC ACC-3′; sequences of MHC-F and MHC-5R were from Hauswaldt et al. (2007). The nested primer MHC-F_F01 (5′-TCA GWR TAR GSS VCA GTG TTA TTA C-3′) was used for gene walking

from all seven species. The PCRs were run for 35 cycles (94°C for 50 s, 60°C for 45 s, and 72°C for 1 min), and a second independent PCR was run on every individual. Eight clones were sequenced from this second run.

Cloning and DNA sequencing

We cloned the PCR products from all reactions into the pGEM® T vector (Promega, Madison, WI, USA) and transformed recombinant DNA into TOP-10 *Escherichia coli* cells (Invitrogen). Cells were grown on Luria agar plates for 18 h at 37°C. We used blue/white screening to randomly choose 8–20 positive clones (from each amplification) to amplify using M13 primers. PCR products were visualized on a 1% agarose gel, and products in the appropriate size range were purified using an alkaline phosphatase–exonuclease reaction. The products were sequenced on an ABI 3100 using Big Dye v3.1 chemistry.

Analysis of nucleotide diversity

We edited the sequence data in SeqMan 7.2 (Lasergene Inc.). We screened each sequence and only included those obtained in at least two clones. We used translated amino acid queries in GenBank (tBLASTx) to confirm MHC class II homology and then aligned the sequences using ClustalW, implemented in MegAlign 7.2 (Lasergene Inc.). We calculated nucleotide distances for the exon 2 and intron 2 regions separately for all seven species in MEGA 4.0 (Tamura et al. 2007) using the Tajima–Nei distance (Tajima and Nei 1984). Amino acid distances (exon 2 only) were calculated with the Poisson correction in MEGA. We used 1,000 bootstrap replicates to calculate standard errors for both distance measures.

Phylogenetic tree reconstruction

To visualize relationships among ranid MHC class IIβ alleles and infer the degree of trans-species polymorphism, we compiled unique exon 2 alleles amplified from cDNA and gDNA in our species with other anuran MHC class IIβ loci. We trimmed our sequences to the 150-bp

length of 30 additional exon 2 published sequences from *R. temporaria*, *Alytes obstetricans*, *B. bombina*, *Bombina pachypus*, *Bombina variegata*, and *X. laevis* (Sato et al. 1993, Kobari et al. 1995, Hauswaldt et al. 2007) identified via BLAST searches in the NCBI database. Two sequences from a caudate amphibian, *Ambystoma mexicanum,* were included as outgroups.

Recombination is a common mechanism underlying the evolution of MHC alleles and can significantly bias phylogenetic analysis (Schierup and Hein 2000); therefore, we first tested for the presence of recombination using permutation analyses in the software Permute (Wilson and McVean 2006; www.danielwilson.me.uk/omegaMap/permute.html). This approach measured the correlation between physical distance along the gene and three measures of linkage disequilibrium: r^2 (Hill and Robertson 1968), D' (Lewontin 1964), and $G4$ (McVean et al. 2002). Significance was determined by permuting the sites 999 times and recalculating the correlation coefficient for each permuted dataset.

We used the longest non-recombining gene segments to estimate a 95% credible set of rooted MHC genealogies for each locus in the software MrBayes 3.1 (Ronquist and Huelsenbeck 2003). For tree building, we used the model of molecular evolution that best fit our data identified from the model selection tool on the Datamonkey server (www.datamonkey.org), which compares the fit of 203 nucleotide substitution models using the Akaike information criterion. We ran two separate analyses in MrBayes for 1×10^7 generations and sampled every 500th generation of the Markov chain. We used Tracer v1.4 to assess the stationarity of model parameters, convergence of model parameters between runs, the number of burn-in samples, and the effective sample sizes for each parameter.

Analysis of selection

We performed two tests of selection on anuran MHC class IIβ exon 2 sequences. We first used the PARRIS method to determine if positive selection was detected in the entire alignment (Scheffler et al. 2006). This method expands on other maximum likelihood methods for detecting positive selection by allowing substitution rates to vary across sites while accounting for recombination by detecting breakpoints when measuring nonsynonymous and synonymous substitution rates. For this analysis, we use the same dataset used for the phylogenetic analysis, but excluded the salamander outgroup sequences (43 sequences, 150 bp of exon 2; ESM Online Resource 2). The PARRIS method test was run on the HyPhy software package hosted at the Datamonkey server.

Second, we tested for residue-specific positive selection across anuran MHC class IIβ exon 2. Using the same dataset (43 sequences, 150 bp of exon 2), we tested for recombination and inferred a tree topology as in the previous phylogenetic reconstruction. This tree was then used as the input tree for selection on particular codons using three different codon-based maximum likelihood methods, all implemented with the Datamonkey server. The most conservative method, single likelihood ancestral counting (SLAC), infers ancestral codon state and then calculates normalized expected and observed nonsynonymous (d_N) and synonymous (d_S) substitutions at each site (Pond and Frost 2005). The fixed effects likelihood (FEL) method directly estimates rates of nonsynonymous and synonymous substitutions at each site, while the random effects likelihood (REL) approach fits a distribution of substitution rates across sites and then infers the rate at which each site evolves (Pond and Frost 2005). To visualize the magnitude of d_N versus d_S, we plotted the d_N minus d_S ($d_N - d_S$) values calculated by FEL. This method is intermediate to the SLAC and REL methods in terms of statistical performance and computational expense (Pond and Frost 2005).

Results

Characterization of MHC IIβ exon 2 in seven ranid species

We isolated 12 exon 2 MHC IIβ chain sequences, corresponding to partial β1 domains, from both genomic and complementary DNA of the seven ranid species we surveyed (GenBank accession nos. HQ025929–HQ025945). Two alleles were identical in exon 2 sequence, but possessed different introns, bringing the total number of unique alleles to 13. There were no differences between the sequences obtained in the first and second PCRs for each animal. We obtained four unique nucleotide sequences from the 14 clones we sequenced from *X. laevis*. All four were identical at the nucleotide level to previously described sequences (GenBank accession nos. BC092157, D50039, EF210753, and NM_001114771).

Our total number of alleles from ranid frogs includes the 187-bp (excluding primers) exon fragments amplified with primers MHC-F and MHC-5R, and the exon and segment of adjacent 3′ intron fragments that were amplified using the primer pair MHC-F and B1intron2_R (Fig. 1). Within species, nucleotide distance of exon 2 varied from 0.062 to 0.197, and amino acid distance ranged from 0.071 to 0.372 (Table 1). Nucleotide distance was highest within *R. clamitans* and lowest in *R. warszewitschii*. The exon 2 alleles we amplified differed by 1–38 nucleotides (and 1–20 amino acids). MHC alleles amplified in this study were named according to the guidelines set forth in Klein et al. (1990) and Ellis et al. (2006), except we used a numerical designation in the second position to describe the putative

Table 1 Comparison of MHC class IIβ exon 2 and intron 2 nucleotide distances (within species mean ± standard error) calculated using the Tajima–Nei distance

D1B refers to only those sequences that were assigned to an orthologous locus based on the similarity of their intron 2 sequences

	Amino acid distance	Nucleotide distance		
	All exon 2	All exon 2	D1B exon 2	D1B intron 2
R. catesbeiana	0.323±0.082	0.155±0.031	–	–
R. clamitans	–	–	–	–
R. palustris	0.071±0.036	0.068±0.019	0.068±0.019	0.015±0.010
R. pipiens	0.130±0.040	0.076±0.018	0.056±0.018	0.015±0.010
R. sylvatica	0.189±0.037	0.130±0.028	0.130±0.028	0.089±0.028
R. warszewitschii	0.111±0.048	0.062±0.018	0.062±0.018	0.015±0.010
R. yavapaiensis	0.146±0.042	0.096±0.017	0.092±0.024	0.046±0.019
All species	0.207±0.040	0.116±0.016	0.101±0.015	0.062±0.013

loci to which each allele belonged (if locus affiliation was not known, an underscore was used in place of a number; Table 2). We used this system to avoid the letters used in *Xenopus* because we could not determine which of our loci were orthologous to those in *Xenopus*.

We found no evidence for pseudogenes in any of our exon 2 sequences (i.e., no deletions, insertions, or stop codons). All the alleles we isolated appeared to represent functional alleles based on the biochemical properties of the residues they contained at sites with known structural importance in the peptide binding region of other vertebrate MHC class IIβ (Brown et al. 1993; Tong et al. 2006). All our alleles contained the two cysteines that form the β1 domain disulfide bond and the three residues (NGT) that are glycosylated. One of the sites involved in salt bonding is also conserved across all alleles (R/K[49]).

Recombination likely plays a role in generating allelic diversity in anurans as it does in other vertebrate groups. However, the three measures of recombination we applied (r^2, D', and $G4$) failed to detect significant recombination present in the region of exon 2 we used for tree building (all $P>0.05$). Therefore, we used the entire 150 bp of exon 2 to generate a phylogenetic tree of anuran MHC alleles. The model selection tool selected the HKY85 model as the best fit for our data, and this model was used in Bayesian inference of topology for subsequent analyses. The phylogenetic tree confirmed the monophyly of anuran alleles relative to those from the salamander, *A. mexicanum* (Fig. 2). The MHC alleles from each genus formed well-supported clades, with no evidence of trans-generic polymorphism. However, within *Rana*, four alleles were found in multiple species, showing evidence of trans-

Table 2 Distribution of MHC class IIβ alleles identified in seven species of ranid frogs (each species is represented by a single individual)

The first four letters are a species identifier, and "D_B" denotes MHC class IIβ and locus affiliation, if known. Symbols denote identical exon 2 alleles present in multiple species

[a] Alleles were only assigned to locus 1 (D1B) or 2 (D2B) if they were amplified with an adjacent intron 2 sequence (or were identical to an allele amplified with an adjacent intron)

[b] Alleles not assigned to a locus are designated D_B

	Alleles			Total no. of unique alleles per individual
	D1B[a]	D2B[a]	D_B[b]	
R. catesbeiana	gDNA: 1 (*Raca-D1B*01*) cDNA: 0	1 (*Raca-D2B*01*) 0	1 (*Raca-D_B*01*) 1 (*Raca-D_B*01*)	3
R. clamitans	gDNA: 1 (*Racl-D1B*01)* cDNA: 1 *(Racl-D1B*01)*	0 0	0 0	1
R. palustris	gDNA: 2 (*Rapa-D1B*01*▲, *Rapa-D1B*02*♦)	0	0	2
	cDNA: 1 (*Rapa-D1B*01*▲)	0	0	
R. pipiens	gDNA: 2(*Rapi-D1B*01*♦, *Rapi-D1B*02*▲)	0	1*(Rapi-D_B*01)*	3
	cDNA: 1 *(Rapi-D1B*01*♦)	0	1 *(Rapi-D_B*01)*	
R. sylvatica	gDNA: 2 (*Rasy-D1B*01*●, *Rasy-D1B*02*▲)	0	0	2
	cDNA: 1 (*Rasy-D1B*01*●	0	0	
R. warszewitschii	gDNA: 2 *(Rawa-D1B*01*■, *Rawa-D1B*02)*	0	0	2
	cDNA: NA	NA	NA	
R. yavapaiensis	gDNA: 2 (*Raya-D1B*01*■ *Raya-D1B*02*)	0	1 (*Raya-D_B*02)*	4
	cDNA: 1 (*Raya-D1B*01*■)	0	1*(Raya-D_B*01*●)*	

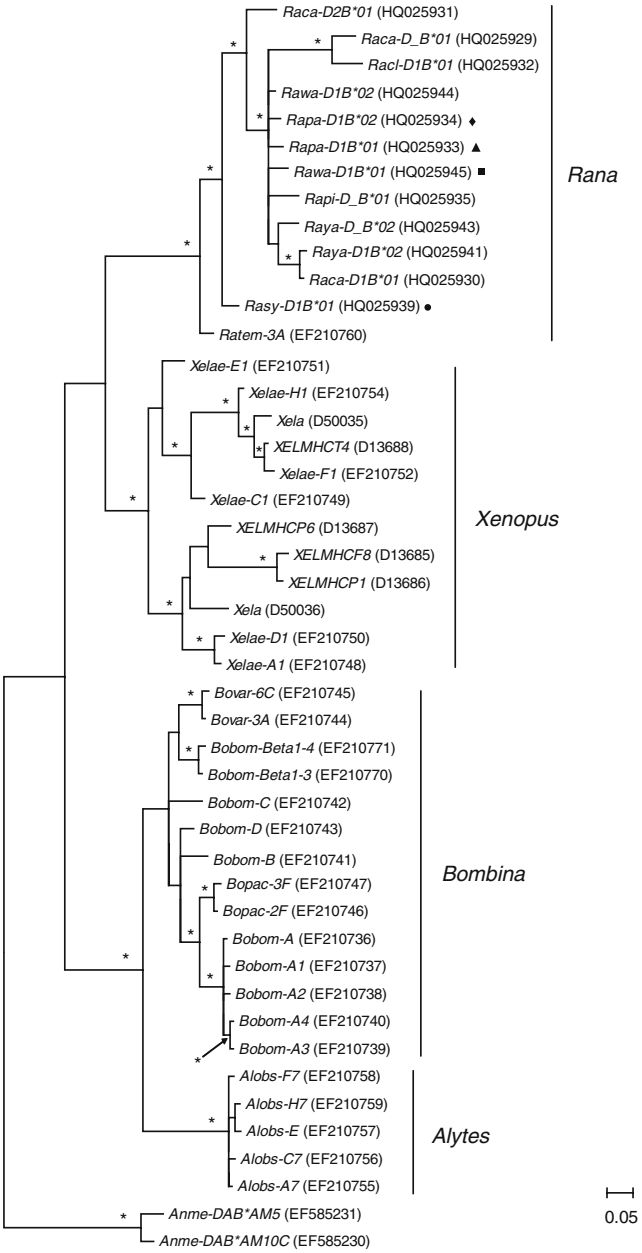

Fig. 2 Phylogenetic tree of anuran exon 2 MHC class IIβ nucleotide sequences constructed with Bayesian inference with two outgroup sequences from the caudate amphibian, *A. mexicanum*. Posterior probabilities above 0.95 are indicated on *branches by asterisks*. *Scale bar* represents 0.05 substitutions per site. *Symbols* mark those alleles that are identical in multiple species (see Table 2 for details). Genera of frogs are listed on the *right*. GenBank accession numbers are given in *parentheses* after each sequence name

species polymorphism in the ranid lineage (Table 2). One D1B exon sequence fell outside the remaining D1B, D2B, and D_B alleles. This result is not surprising because the structural and functional homology among class II paralogs across vertebrates may result in groups that are not composed of alleles from a single locus.

Isolation of MHC IIβ intron 2 in ranids

Gene walking assays resulted in positive MHC class IIβ clones from three species, *R. yavapaiensis*, *R. clamitans*, and *R. catesbeiana*. The first 250 bp of coding sequence were highly similar (~49–67% amino acid sequence identity) to MHC class IIβ cDNA clones of *X. laevis* and *S. tropicalis*. Ten base pairs were perfectly conserved adjacent to the 3′ boundary of exon 2 in nearly all of our clones, but beyond this region, the ranid intron 2 sequences diverged from *Xenopus* and *Raca-D2B*01* (ESM Online Resource 3).

The longest stretch of intron 2 was obtained in *R. yavapaiensis* (GenBank accession no. HQ025941), extending ~420 bp past the intron/exon boundary, followed by *R. clamitans*, which extended ~190 bp (HQ025932). Two different *R. catesbeiana* intron 2 sequences were obtained from gene walking (HQ025930 and HQ025931). One round resulted in a sequence that extended 30 bp past the intron/exon boundary and was highly similar to the sequences of *R. yavapaiensis* and *R. clamitans*. The other round of gene walking resulted in a unique *R. catesbeiana* intron 2 sequence that could not be aligned unambiguously with either the *R. yavapaiensis* or *R. clamitans* introns beyond the first 17 bp. The ranid intron 2 sequences clearly grouped into two clusters, one with >80% similarity and the other containing the single *R. catesbeiana* sequence. Based on this result, we assigned the three closely related sequences from the three species as a putative single locus (D1B) and the remaining *R. catesbeiana* sequence to a second locus (D2B).

Using these initial gene walks as templates, we designed a reverse intron primer to pair with the MHC-F primer that had an annealing site within the exon 2 and amplified both partial exon 2 and intron 2 from all species (Fig. 1). The length of this fragment containing exon 2 and intron 2 amplified with MHC-F and B1intron2_R varied from 376 to 391 bp (excluding primers) because of nucleotide insertions/deletions present in the intron 2 of some species (ESM Online Resource 3).

Genetic variation of MHC IIβ exon 2 and intron 2

Overall, exon 2 of MHC class IIβ exhibited higher levels of variation than intron 2 (Table 1). In the most extreme case, the two exon 2 sequences of *R. palustris* had more than four times the evolutionary distance between them than did the two intron sequences. While the average nucleotide distance of exons ranged from 0.062 to 0.197 for exon 2 across all species, the mean intron distance across species ranged from 0.015 to 0.089.

Our survey of alleles present in cDNA and gDNA yielded complex results (Table 2). We expected that we

might recover different alleles in cDNA versus gDNA (using MHC-F and MHC-5R) since some alleles may only be expressed in particular tissues and therefore cDNA may only contain a subset of the alleles present in the genome. However, every allele present in cDNA should have been recovered in the gDNA of that individual, but this was not the case (Table 2). One allele was only amplified from cDNA and not gDNA with the primers MHC-F and MHC-5R (*Raya-D_B*01*); this phenomenon could possibly have been caused by differing abundances of each allele in the template gDNA versus cDNA.

We suspect that the lack of recovery of certain alleles could also be caused by primer mismatch, a phenomenon which we investigated in certain alleles that contained the MHC-5R primer annealing site. We found that alleles recovered with MHC-F and B1intron2_R generally did not amplify in reactions using the two exon primers (MHC-F and MHC-5R) when they had a high number of base pair mismatches in the MHC-5R annealing site. The primer binding sites of the two alleles amplified with adjacent intron sequence from *R. palustris* illustrate this point. Allele *Rapa-D1B*01* has two positions that differ from the primer, while *Rapa-D1B*02* has three (ESM Online Resource 3). As seen in Table 2, *Rapa-D1B*01* was recovered in the reactions using the exon primer pair, MHC-F and MHC-R, while *Rapa-D1B*02* was not. These results show that primer composition influences what alleles are recovered.

Selection on MHC II β chain B1domain residues in anurans

The PARRIS method found strong evidence for selection acting on the alignment of 43 anuran MHC IIβ sequences ($P<0.01$). The three site-by-site methods (SLAC, FEL, and REL) each found evidence for selection acting on particular residues (Fig. 3). The peptide-binding regions (PBR) are not yet known in frogs; therefore, PBR sites were putatively identified using the models of Brown et al. (1993) and Tong et al. (2006) defined for human MHC locus HLA-DRB. We mapped evolutionarily conserved residues based on the results in Kaufman et al. (1994). In the 50 amino acid alignment, we identified 14 putative PBR sites and 36 non-PBR sites, with nine being evolutionarily conserved. The REL method identified many of the sites congruent with our expectations (i.e., PBR sites being under positive selection and conserved sites being under negative selection because mutations at these sites are likely to result in deleterious changes to protein structure and thus would be eliminated from the population), but also classified many other sites as well (Fig. 3c). SLAC was the most conservative method, identifying three residues as under positive selection and eight under negative selection, but 8 of the 11 total sites under selection mapped onto evolutionarily conserved or PBR residues (Fig. 3a). In contrast, REL predicted 27 sites that had experienced significant selection, but 11 of these did not map onto any putatively important residues (Fig. 3c). The $d_N - d_S$ plot calculated by FEL (which predicted an intermediate number of sites under selection compared to SLAC and REL methods) illustrated the variation in specific $d_N - d_S$ values across codons in the β1 domain (Fig. 4). Notably, the two sites identified to have the highest $d_N - d_S$ (positions 6 and 49) are both putative PBR sites and were both found to be under positive selection using all three analytical methods. Concordantly, the two sites identified to have the lowest $d_N - d_S$ (positions 3 and 20; Fig. 4) are both evolutionarily conserved sites and were both found to be under negative selection using all three analytical methods (Fig. 3).

Discussion

We amplified MHC class IIβ alleles (β1 domain) from the genome and transcriptome of ranid frogs. In total, we indentified 13 alleles from seven individuals. Within-species β1 domain nucleotide distances (0.062–0.155) appeared to be comparable to that documented in other amphibians (Hauswaldt et al. 2007; May and Beebee 2009; Zeisset and Beebee 2009). For example, in over 200 individuals of *R. temporaria*, nucleotide diversity was 0.077 (Zeisset and Beebee 2009), while in a similar number of newts, diversity was 0.096 (Babik et al. 2008). We also found trans-species polymorphism within the exon 2 regions among *Rana*, with the caveat that additional substitutions may exist in other exons of these alleles, rendering them closely related rather than identical. Trans-species polymorphism has been documented in the class IIβ genes of another amphibian genus, *Ambystoma* (Bos and DeWoody 2005), and in the class I genes of *Xenopus* (Bos and Waldman 2006) and is likely to be characteristic of the MHC genes of most amphibian groups.

Multiple factors are likely promoting (and possibly restricting) allelic diversity in ranid frogs. In our dataset, we found no evidence of recombination, a known mechanism for generating MHC diversity in class IIβ. However, we attribute this result to the shortness of the fragment we amplified instead of a true absence of intergenic recombination in anuran class IIβ. Recombination has been documented in *Xenopus* MHC classes I and II (Bos and Waldman 2006), and thus, we expect that recombination would be detected if our analyses had included the entire coding region or if we had characterized more alleles in a

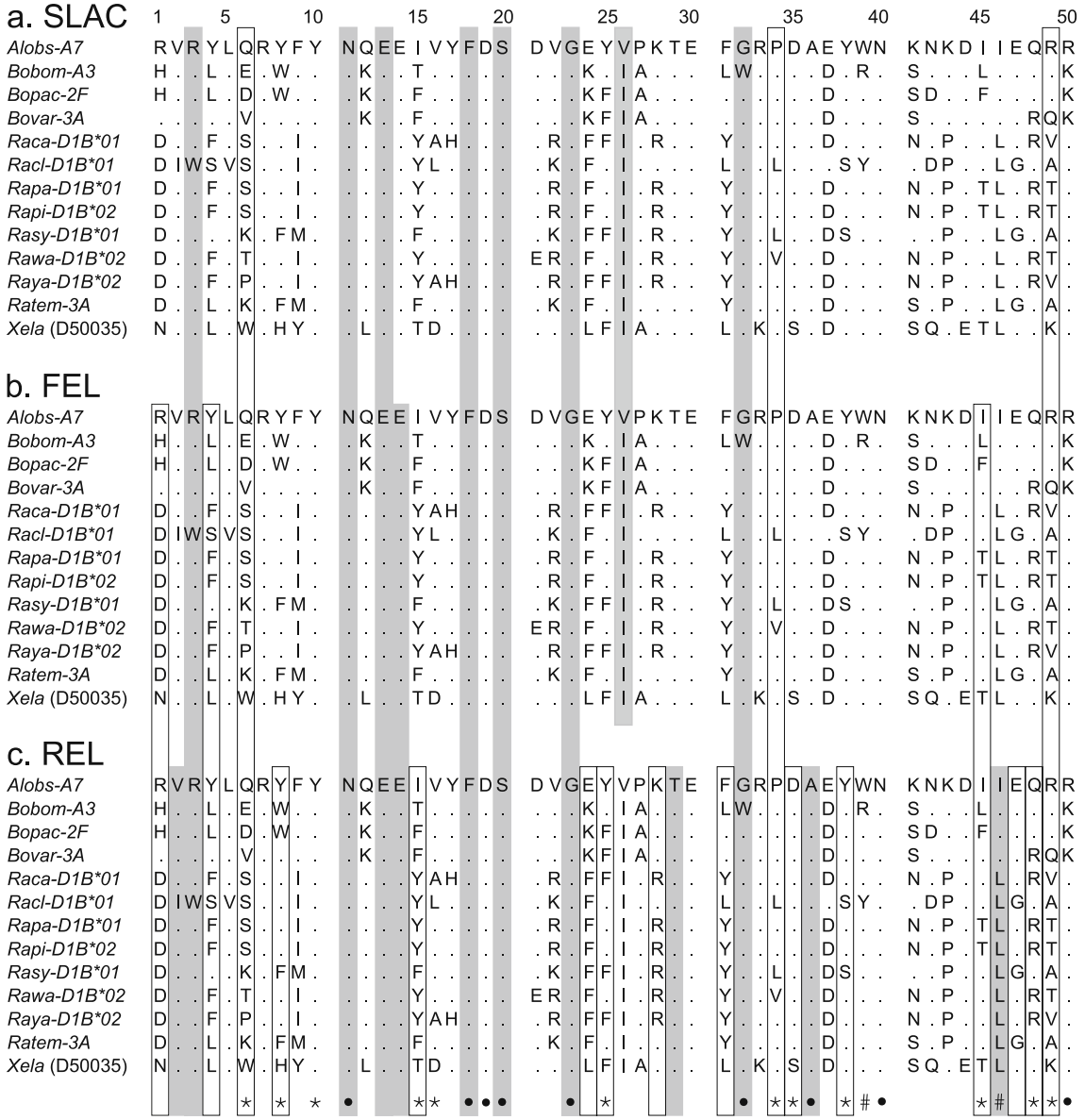

Fig. 3 Alignment of the β1 domain of MHC class II of anurans illustrating selection on amino acid residues. Sites under significant selection were determined by an analysis of 43 anuran sequences (see text for details); a subset containing representative sequences from each species is shown here. Amino acid residues found to be under positive selection are indicated by *open boxes* and those under negative selection are *shaded*. The significant signature of selection was determined by single likelihood ancestral counting (**a**) fixed effects likelihood (**b**), or random effects likelihood (**c**). *Dots below the alignments* denote evolutionarily conserved residues described by Kaufman et al. (1994). Residues involved in peptide binding in human MHC class II β1 domain are marked with *asterisks* (identified by Brown et al. 1993 and/or Tong et al. 2006). Residues that are both involved in binding and evolutionarily conserved are noted by *sharp signs*

larger number of individuals. In addition to recombination, the positive selection we detected is indicative of a high degree of MHC polymorphism in anurans. This diversity may be maintained by a number of mechanisms, including balancing selection, heterozygote advantage, and frequency-dependent selection (Hughes and Nei 1992; Hedrick and Kim 2000). In particular, diversifying selection appears to be acting on residues involved with antigen binding. However, some positively selected sites in frogs were not found to be involved in peptide binding in equivalent human MHC class II proteins, a phenomenon also documented in caudate amphibian MHC class II (Richman et al. 2007). It is possible that some functionally important residues in the frog MHC PBR are different than those in humans. Although the site-by-site selection detection methods identify high d_N/d_S ratios, substitutions

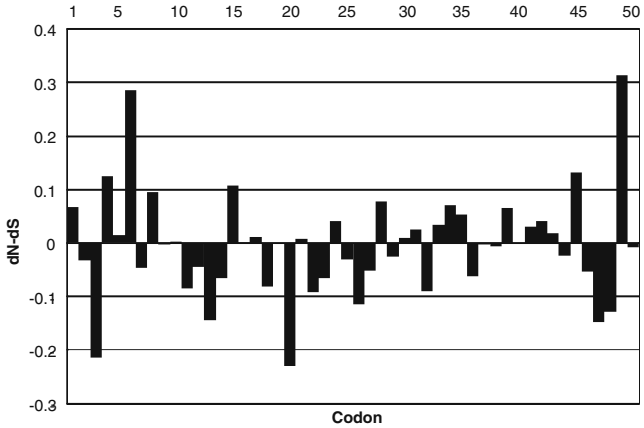

Fig. 4 MHC class II β1 domain by site values for normalized d_N minus d_S ($d_N - d_S$), calculated with FEL. When $d_N - d_S=0$, site is neutrally evolving; $d_N - d_S > 0$, site is positively selected; $d_N - d_S < 0$, site is negatively selected. Codon numbering matches that of Fig. 3

at some of these sites may not be causing adaptive changes in the protein (Nozawa et al. 2009). These confounding factors will be easier to disentangle once more is known about the molecular structure of frog MHC proteins.

We present two lines of evidence that ranid frogs have at least two class IIβ loci. First, we amplified two divergent intron sequences from a single *R. catesbeiana* individual. Although one was similar to intron sequences from other ranids, the other showed little similarity to either the other ranid introns or the *X. laevis* intron. Secondly, we uncovered three or four alleles from the genome of the *R. yavapaiensis*, *R. pipiens*, and *R. catesbeiana* individuals. These numbers are consistent with the data available from *R. temporaria*, *B. bombina*, *B. calamita*, and *X. laevis*, suggesting frogs have at least two MHC class IIβ genes (Sato et al. 1993; Hauswaldt et al. 2007; May and Beebee 2009; Zeisset and Beebee 2009). However, we cannot rule out that frogs in the genus *Rana* have more than two loci. Individuals may possess alleles from divergent lineages that did not amplify with our primers and/or some of the exon alleles we amplified that were not assigned as alleles of locus "1" may belong to multiple other loci.

We found that primer sequence influences which alleles are amplified during a particular PCR (PCR bias). Working with multiple loci has long been recognized as an issue in MHC studies (Babik 2009), and the total number of alleles obtained from a particular individual using degenerate PCR must be interpreted with caution. Non-amplifying alleles can influence population parameter estimates and bias inferences of number of loci and allelic diversity. This type of PCR bias has been observed in many other studies of MHC genes; for example, PCR bias was documented in guppy MHC class II where some alleles were cloned more frequently than others (van Oosterhout et al. 2006). In our study, we hypothesize that the primer pair that sat within the exon may have been more prone to bias because of the greater number of potential templates present in each gDNA sample due to the presence of multiple class IIβ loci. Our experiment showed that in *Rana*, the primer pair targeting a single locus (MHC-F and B1intron2_R) was less prone to allelic amplification bias than the primer pair consisting of two degenerate primers designed to target all exon 2 sequences present in the genome (MHC-F and MHC-5R). But, we cannot completely rule out that some *Rana* individuals may have divergent D1B alleles that are not amplifying even with the single locus primer set.

Intron sequences obtained from multiple species in our study show that the development of the primer pair MHC-F and B1intron2_R allowed us to amplify alleles from an orthologous locus in our focal species ranid frogs. Across all species, the mean number of synonymous substitutions in the exons (0.140±0.038) was twice as high as the mean number of substitutions per site in the introns (0.062± 0.013), a result consistent with the hypothesis that recombination between alleles has homogenized these introns. Over evolutionary time, an exon may be maintained by balancing selection, but drift and recombination can lead to the fixation of a particular adjacent intron sequence, a pattern associated with introns that were locus-specific in primates (Cereb et al. 1997). In addition, these primers consistently amplified the fragment of exon 2 and intron 2 from divergent species in the genus *Rana*; thus, these primers will likely amplify orthologous MHC loci from a number of *Rana* species. Our amplifications using cDNA templates also show that alleles from this putative locus are transcribed, and therefore, functional protein products are likely encoded by this locus. However, HLA -DRB6 is a pseudogene that is still transcribed into predominately non-coding RNA (Mayer et al. 1993; Fernandez-Soria et al. 1998), and thus, full-length cDNAs will be necessary to confirm that these alleles contain a complete coding region characteristic of MHC IIβ loci.

Our development of single-locus MHC class IIβ primers now enables population genetic-level comparisons of diversity and identification of specific alleles that might participate in defending against emerging diseases in declining anurans. Species in the family Ranidae demonstrate the third highest rate of decline among all anuran groups (IUCN 2010), and many of these declines are attributable to chytridiomycosis (Bradley et al. 2002). It is also hypothesized that frog populations may be negatively impacted by ranaviral disease (e.g., Green et al. 2003). Despite extensive analyses of the patterns of pathogen-associated species declines (Pounds et al. 2006; Lips et al. 2006), the relevance of host immunogenetics to susceptibility remains largely unexplored. Candidate gene studies

targeting functional immunity genes have been conducted in other non-model vertebrate systems and show that MHC allelic composition can be associated with parasite resistance in host populations (reviewed in Bernatchez and Landry 2003). In some species, MHC heterozygotes have higher survival than homozygotes after exposure to pathogens (Hedrick et al. 2001; Arkush et al. 2002), while in others, survival after exposure to a specific disease is correlated with particular MHC alleles (Paterson et al. 1998; Langefors et al. 2001; Grimholt et al. 2003). For example, infection with ranavirus is associated with particular MHC class I alleles in *R. temporaria* (Teacher et al. 2009). Our study will stimulate more studies such as these that further characterize how immunogenetic variability influences disease susceptibility associated with amphibian declines.

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