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Evolutionary dynamics of an expressed MHC class IIβ locus in the Ranidae (Anura) uncovered by genome walking and high-throughput amplicon sequencing

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ABSTRACT

The Major Histocompatibility Complex (MHC) is a genomic region encoding immune loci that are important and frequently used markers in studies of adaptive genetic variation and disease resistance. Given the primary role of infectious diseases in contributing to global amphibian declines, we characterized the hypervariable exon 2 and flanking introns of the MHC Class IIβ chain for 17 species of frogs in the Ranidae, a speciose and cosmopolitan family facing widespread pathogen infections and declines. We find high levels of genetic variation concentrated in the Peptide Binding Region (PBR) of the exon. Ten codons are under positive selection, nine of which are located in the mammal-defined PBR. We hypothesize that the tenth codon (residue 21) is an amphibian-specific PBR site that may be important in disease resistance. Trans-species and trans-generic polymorphisms are evident from exon-based genealogies, and co-phylogenetic analyses between intron, exon and mitochondrial based reconstructions reveal incongruent topologies, likely due to different locus histories. We developed two sets of barcoded adapters that reliably amplify a single and likely functional locus in all screened species using both 454 and Illumina based sequencing methods. These primers provide a resource for multiplexing and directly sequencing hundreds of samples in a single sequencing run, avoiding the labour and chimeric sequences associated with cloning, and enabling MHC population genetic analyses. Although the primers are currently limited to the 17 species we tested, these sequences and protocols provide a useful genetic resource and can serve as a starting point for future disease, adaptation and conservation studies across a range of anuran taxa.

1. Introduction

Genes of the Major Histocompatibility Complex (MHC) are among the most polymorphic loci in the vertebrate genome (Kelley et al., 2005) and play an important role in pathogen resistance (Madsen and Ujvari, 2006) as well as mate choice (see Milinski, 2006 for a review). The MHC genomic region includes class I genes, which typically recognize intracellular pathogens and self/nonself peptides, and class II genes that are more often associated with extracellular pathogens (Bevan, 1987). Both class I and class II MHC genes are a central part of the vertebrate adaptive immune response because they encode glycoproteins which recognize and bind to pathogen peptides and subsequently present them to T-cells for removal (Bevan, 1975). Because higher levels of MHC variation allow more different pathogens to be recognized by the immune system, balancing selection can promote the maintenance of large numbers of MHC alleles in populations over extensive periods of

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evolutionary history. Due to this high variability and important role in immunity, MHC genes are ideal markers for measuring the genetic viability and health of populations, particularly in the context of disease (Trowsdale, 2011). Consequently, MHC coding regions are frequently used markers for studies on adaptive genetic variation (Bernatchez and Landry, 2003; Garrigan and Hedrick, 2003; Sommer, 2005), sexual selection (Ekblom et al., 2010; Kamiya et al., 2014), and general assessments of genetic diversity (Sommer, 2005).

MHC studies often focus on the peptide-binding region (PBR) of the genes, which encode the area of the protein that recognizes and attaches to antigens and presents them to the T-cells to trigger the acquired immune response. Within class II MHC genes the majority of amino acids comprising the PBR are located in exon 2 of the gene (Brown et al., 1993; Tong et al., 2006), whereas the class I PBR is coded for in both exons 2 and 3 (Bjorkman et al., 1987). High variability of MHC loci makes them useful and interesting genes for functional genetic and evolutionary analyses, but this same property also makes them difficult to work with in non-model organisms with limited available genetic information (Babik, 2010). Several factors hamper MHC genotyping, including inter- and intralocus gene duplications and deletions (Kelley et al., 2005; Klein et al., 1993), high nucleotide diversity for primer development (Kiemnec-Tyburczy et al., 2010) and the difficulty of obtaining phased genotypes, also due to high variability (Guo et al., 2006). An additional problem with using exon-based primers in conserved regions is that these sequences are often shared across gene copies and thus do not allow for single-locus genotyping (Meyer-Lucht et al., 2016).

Reliably screening populations and species for important markers of disease resistance such as MHC loci is an increasingly important aspect of conservation management (Altizer et al., 2003) in order to both understand potential response to a disease introduction and to predict adaptive potential of populations. Conservation geneticists frequently use only neutral genetic variation in status assessments, and in doing so do not take into account adaptive variation (Hedrick, 2001), which may sharply differ from neutral loci (Aguilar et al., 2004; Jarvi et al., 2004). Increased knowledge and availability of adaptive genetic variation, such as the genes of the MHC, can thus influence important management decisions and help conserve the adaptive potential of endangered species (Sommer, 2005). This is particularly important for endangered species and small or isolated populations that have limited genetic variation due to historical bottlenecks (Frankham, 2005; Hoelzel, 1999), resulting in a higher susceptibility to disease (O'Brien et al., 1985; Spielman et al., 2004), which can in turn provide the proximate cause of extinction.

Amphibians are the most threatened group of vertebrates (Hoffmann et al., 2010; Rollins-Smith, 2017), but they continue to be understudied (Hecnar, 2009) and have limited genomic resources (Koepfli et al., 2015) to help inform conservation management. Emerging infectious diseases have played a significant role in amphibian declines, particularly the fungus *Batrachochytrium dendrobatidis* (Bd; Berger et al., 2016) and the recently discovered *B. salamandrivorans* (Bsal; Martel et al., 2013), as well as several clades of Iridoviruses in the genus *Ranavirus* (Rv; Miller et al., 2011). There are strong differences in disease susceptibility among species (Lips, 1999; Searle et al., 2011; Woodhams et al., 2007) as well as within species (Savage and Zamudio, 2011). These differences can have both environmental (Bustamante et al., 2010; Piotrowski et al., 2004; Pounds et al., 2006) as well as genetic (Berger et al., 2005; Savage and Zamudio, 2011; Tobler and Schmidt, 2010) mechanisms. MHC class II alleles are directly associated with Bd susceptibility in both lab trials (Bataille et al., 2015; Savage and Zamudio, 2011) and field studies (Savage and Zamudio, 2016) and class I alleles are associated with Rv infection status (Teacher et al., 2009). MHC genes are therefore strong candidate loci for research regarding the relationship between host genetics and disease resistance in amphibian disease systems (Carey et al., 1999), and can also be important markers in more applied conservation studies such as in captive breeding and head starting programs (Gratwicke et al., 2016; Ujvari and Belov, 2011). Here, we focus exclusively on class II characterization because MHC expression in amphibian larvae, the life stage that is most susceptible to Rv, is largely restricted to class II alleles (Flajnik et al., 1986), and because class II molecules are more typically involved in recognizing fungal pathogens such as Bd (Richmond et al., 2009).

Given the potential benefits of increased knowledge of MHC variation to inform conservation managers and aid studies of adaptive variation, we characterized the complete MHC class IIβ exon 2 and intronic flanking regions for a variety of species in three well-studied anuran genera, *Lithobates*, *Rana* and *Pelophylax,* to provide genetic resources for future studies (see Hillis and Wilcox, 2005 for information on the subdivision of the original *Rana* genus into several genera). Current published genetic information on MHC class II introns in this clade is limited to approximately 137 base pairs (bp) of intron 2 for the species *L. yavapaiensis*, *L. warszewitschii*, *L. pipiens*, *L. palustris* and *L. catesbeianus*, whereas no information is available concerning intron 1 of these genera and the first 16 bp of exon 2 (Kiemnec-Tyburczy et al., 2010). Here, we generate and characterize novel sequence information of approximately 357 bp of intron 1 as well as 16 bp of exon 2 for 17 new species, and add information for 14 new species for the ~137 bp already known from intron 2 (Kiemnec-Tyburczy et al., 2010). We present primers that amplify the complete exon and intronic flanking regions and are ideal for Sanger sequencing, as well as barcoded fusion primers that amplify 97% of exon 2 and can be readily used for multiplexing on 454 and Illumina sequencing platforms. These new resources enable functional genetic studies of adaptation, mate choice and disease resistance across at least three diverse anuran genera.

2. Methods

2.1. Genome walking, primer design and Sanger sequencing

Toe clips from 11 of our focal species were non-lethally collected from natural amphibian populations following permitted institutional guidelines and stored in 95% EtOH (Table 1). For the remaining six species, tissues were subsampled from liver samples stored at the Division of Amphibians and Reptiles at the Smithsonian National Museum of Natural History. Both toe and liver samples were extracted using DNeasy blood and tissue kits (Qiagen, Valencia, CA) following manufacturer's guidelines. All DNA extractions were eluted in 200 μl of AE buffer and stored at −20 °C.

We performed genome walking to gather information on the MHC class IIβ complete 5' exon 2 and flanking intron 1 using the Universal Genome Walking kit (Clontech, Palo Alto, CA). We followed kit guidelines except for the gel purification step, which was done with 2X Sera-Mag Speed Beads (Thermo Fisher Scientific, Waltham, MA). Libraries were amplified using long reverse primers of around 27 base pairs (GW_01, GW_02, GW_03, see Table 2) that were designed in conserved parts of exon 2. Primer locations were based on an alignment of 33 GenBank (NCBI) sequences from *Lithobates yavapaiensis* alleles (JN638850-JN638882) and 5 sequences obtained by Sanger sequencing using tissue samples from *Lithobates chiricahuensis* and previously developed primers (Kiemnec-Tyburczy et al., 2010). The first round of genome walking was performed on one *L. chiricahuensis* DNA sample using four different restriction enzymes (DraI, EcoRV, PvuII and StuI) to find a

Table 1
List of species with sample source localities. Best primers for both Sanger and amplicon sequencing are listed with notes on their efficiency on tested species. * only 1 allele was found with 454 sequencing because the second allele from Sanger sequencing is based on a SNP in the intron 1.

Species	Source	Location	Longest Sanger sequence primer pair	Amplicon sequencing primer pair	Platforms tested	Proportion successful (# alleles)
Lithobates chiricahuensis	field	Arizona, USA	ForB - B1intron2_R	ForN - RevA	454	499/575 (5)
Lithobates catesbeianus	field	Arizona, USA	ForB - B1intron2_R	ForN - RevA	454	3/3 (4)
Lithobates sylvaticus	field	Maryland, USA	ForB - B1intron2_R	ForN - RevWood	454	342/396 (24)
Lithobates virgatipes	USNM332745	North Carolina, USA	ForB - RevA	ForN - RevA	454	1/1 (1) *
Lithobates palustris	USNMFS-PALU1	Unknown	ForB - RevA	ForN - RevA	454	1/1 (2)
Lithobates vaillanti	USNM348437	Panama	ForB - RevD	ForN - RevWood	x	x
Lithobates spenocephalus	USNM547948	North Carolina, USA	ForB - RevD	ForN - RevA	454	1/1 (1)
Lithobates forreri	USNM534222	Honduras	ForB - RevE	ForN - RevA	x	x
Lithobates clamitans	USNMFS-CLAM4	Unknown	ForB - RevA	ForN - RevA	x	x
Lithobates yavapaiensis	field	Arizona, USA	ForB - B1intron2_R	ForN - RevA	454	67/296 (6)
Lithobates blairi	field	New Mexico, USA	ForB - B1intron2_R	ForN - RevA	454	10/10 (5)
Rana temporaria	field	Germany	ForB - RevA	ForN - RevA	x	x
Rana arvalis	field	Sweden	ForB - B1intron2_R	Elf_1 - Elr_2	Illumina	210/240 (57)
Rana macrocnemis	field	Turkey	ForB - B1intron2_R	ForN - RevA	x	x
Rana pyrenaica	field	Spain	ForB - RevA	ForN - RevA	x	x
Rana iberica	field	Spain	ForB - RevA	ForN - RevA	x	x
Pelophylax lessonae	field	Sweden	ForB - B1intron2_R	ForN - RevF	x	x
Odorrana tormota	Shu et al., 2013	China	ForB - B1intron2_R	x	x	x

Table 2
List of primers used for Sanger sequencing in this study. GW primers were used for the upstream primer walking, all other primers are for PCR amplification of the MHC exon 2 (see Fig. 1 for a schematic illustration of primer locations).

Primer Name	Sequence (5'-3')	Source
MHC-F	CCSCAGAKGATTWCGTGWMTCA	Hauswaldt et al., 2007
MHC-5R	TGTCTGCAGACTGTYTCCACCHCAGCC	Hauswaldt et al., 2007
B1intron2_R	CACATAATCCAGTAGTARAAAGYCACC	Kiemnec-Tyburczy et al., 2010
GW_01	GACATCCTCCGTCCCGTTCCGGTAATA	this study
GW_02	TGTCGAAGTACACAWACTCCTCCTGATT	this study
GW_03	CCGGTACTCACATTTCCGCTCTATGGC	this study
ForB	TGTAAGTGATACAATGTTATCAGATGT	this study
ForN	GTTCTCTCCCCGCAGATGATTTC	this study
RevA	GCATAGCAGACGGAGGAGT	this study
RevC	TCATAGGAGATCTGMGAGCATAGCA	this study
RevD	AGGTAAGAGAGTGATCTTCCTTGAATGA	this study
RevF	GCTTASTAGCCAGGTGCAGT	this study
RevWood	GGAGATCTCCGAGCCTAGCA	this study
Elf_1	GAGGTGATCCCTCCAGTCAGT	this study
Elr_2	GCATAGCAGACGGAGGAGTC	this study

restriction site that was at a convenient distance for both PCR amplification and for Sanger sequencing.

An additional 8 *L. chiricahuensis* and 4 *L. yavapaiensis* samples were selected for genome walking using only PvuII. To reduce kit costs, extra PvuII and T4 ligase was ordered separately (New England Biolabs, Ipswich, MA) and adapter concentration was halved. PCR product was sequenced using an ABI 3130 automated DNA sequencer (Applied Biosystems, Foster City, CA) and the sequences were manually checked and aligned in Geneious 9.1 (Kearse et al., 2012). Based on these 12 samples an array of forward primers was developed using the primer3 software package integrated into Geneious 9.1 (Kearse et al., 2012; Rozen and Skaletsky, 1999). Forward primers were used in conjunction with reverse primer B1intron2_R (Kiemnec-Tyburczy et al., 2010) and other reverse primers that we developed based on the alignment of *L. yavapaiensis* and *L. chiricahuensis* alleles described above. Different combinations of primers were used on all other species to maximize the amount of sequence information generated per species (Table 1).

All amplicons were sequenced in both the forward and reverse direction. We edited sequences by eye and removed whole or parts of sequences with ambiguous electropherograms. Only sequences with clean base calls for the complete exon region were retained. Heterozygotes were used for primer design but were excluded from the alignment due to problems associated with ambiguous base pairs in downstream analysis and the small sample size per species, which did not allow for accurate phasing of genotypes.

2.2. Amino acid properties

We removed duplicate alleles and added two outgroup species, *Odorrana tormota* (Shu et al., 2013) and *Nanorana parkeri* (Sun et al., 2015), that also have published MHC class IIB introns (GenBank ID: KC414849 and PRJNA243398). The final dataset was aligned using 16 iterations in MUSCLE as implemented in Geneious 9.1 (Kearse et al., 2012). Exon-intron boundaries were assigned using *Xenopus laevis* (GenBank ID: D50039), corresponding PBRs were identified from the Brown model (Brown et al., 1993) and conserved codons were assigned following Kaufman et al. (1994). We also included the results of the Tong et al. (2006) modelling study as a second model for the PBR to detect potential codons outside the canonical representation of the PBR. Both nucleotide diversity (π) and Tajima's D were calculated in MEGA 7 (Kumar et al., 2016). Sequences were translated with Geneious 9.1 and we scanned the amino acid alignment for the presence of stop codons and inconsistencies by eye.

2.3. Phylogenetic reconstruction

All phylogenetic analyses were performed independently on the exon and the combined intron sequences in order to be able to compare their evolutionary histories. PartitionFinder 1.1 (Lanfear et al., 2012) was used on the exon and separately on the concatenated intron sequences, allowing only for substitution models that were compatible with MrBayes and using unlinked branch lengths. The corrected Akaike Information Criterion (AICc; Hurvich and Tsai, 1993) was applied to determine the best model for both genealogies. We allowed partitioning of the exon across all three codon positions, and the intron 1 and intron 2 sequences were allowed to be partitioned for the concatenated intron alignment. We used MrBayes 3.2 (Ronquist et al., 2012) to build genealogies using the best model for each alignment over ten million generations with a

sampling frequency of 500 generations. The resulting tree was built excluding a burn in of 25% and figures were edited in FigTree 1.4.2 to include posterior probabilities and clade information.

2.4. Co-phylogeny analyses

A separate dataset that included only one allele per species was constructed for MHC intron and exon co-phylogenetic analysis and comparisons of MHC intronic and exonic genealogies with a mitochondrial phylogeny. The most complete MHC Sanger sequence per species was selected, and when numerous complete alleles were present we used a random number generator to select one for inclusion. For these same individuals, we also sequenced a fragment of 658 bp of the cytochrome oxidase subunit I (COI) using published primers (Folmer et al., 1994) or we retrieved sequences from GenBank (see supplementary material Table A1). Sequences were aligned with MUSCLE for 16 iterations and phylogenetic analysis was performed with FastTree 2.1.5 (Price et al., 2010) using default settings as implemented in Geneious 9.1 (Kearse et al., 2012). Co-phylogenies were built using the tanglegram function from the dendextend package in R (Galili, 2015) using ultra-metric trees and assigning *Pelophylax lessonae* as outgroup. We also quantified differences among gene trees using branch-score differences and CADM values (Congruence Among Distance Matrices) from the R packages phangorn and APE (Paradis et al., 2004; Schliep, 2011).

2.5. Selection analyses

Using the Hypothesis testing using Phylogeny (HyPhy; Kosakovsky Pond et al., 2005) datamonkey server (www.datamonkey.org) we tested for positive (or diversifying) and negative (or purifying) selection using six different methods (Delport et al., 2010). Because recombination can lead to inaccurate selection analyses, prior to performing tests of selection we first tested for recombination using both GARD (Genetic Algorithms for Recombination Detection) and SBR (Single Breakpoint Recombination; Kosakovsky Pond et al., 2006a). We used all five codon-based maximum likelihood tests of selection: FEL (Fixed Effects Likelihood), SLAC (Single Likelihood Ancestry Counting), REL (Random Effects Likelihood), iFEL (internal-FEL) and FUBAR (Fast Unconstrained Bayesian AppRoximation), as well as PARRIS (PARtitioning approach for Robust Inference of Selection) to detect gene-wide selection (Delport et al., 2010). For these analyses, we used the exon-based MrBayes tree and the nucleotide substitution model that was selected automatically by HyPhy. Significance was set at a *P*-value of 0.05 unless stated otherwise. For all five codon-based methods, we only included the codons that were found to be under selection by at least two out of five methods, as concordant results across methods increase the confidence that codons are truly under selection. We also tested for positive selection using MEME (Mixed Effects Model of Evolution), which does not allow testing for negative selection, and we therefore report it separately from the other five methods. As GenBank currently holds thousands of MHC class II sequences, we compared the codons under selection that were not within the PBR to a previously published representative dataset of 55 vertebrate alleles that includes 23 amphibians (Lillie et al., 2016) to determine if our results were amphibian specific or vertebrate-wide. Corresponding sequences were retrieved from GenBank (except for AF209116 as it has a frameshift and likely is an error; Laurens et al., 2001), and the coding regions were aligned using 16 iterations in MUSCLE using the translation option in Geneious 9.1 (Kearse et al., 2012). Alignments were split into an amphibian and non-amphibian group and the dN/dS values were calculated in MEGA 7 with default settings (Kumar et al., 2016).

2.6. High-throughput sequencing resources

Fusion primers were designed to use high-throughput multiplexed amplicon sequencing to genotype many samples in parallel and to enable phasing of the different alleles for heterozygous individuals without cloning. Primers were designed as close to the exon boundaries as possible in order to keep sequence length within the limits of the majority of reads from a 454 Junior run. Barcodes were designed that excluded homopolymers within the barcode as well as with the two neighbouring bases of the primer and adapter. We included at least two base pair differences between all barcodes so that one PCR or sequencing error per barcode would not alter accurate assignment of reads to samples (Faircloth and Glenn, 2012). Barcodes were inserted into both the forward and reverse primer to allow multiplexing of samples with unique combinations of forward and reverse barcodes. Fusion primer performance was tested on a subset of species on a 454 amplicon run using titanium chemistry on an in-house 454 FLX and 454 Junior (Center for Conservation Genomics, Washington D.C., USA). To negate any potential PCR bias, all samples were amplified in triplicate with each replicate using a unique barcode. Replicates were randomized across PCRs as well as sequencing runs (Sommer et al., 2013). Samples were pooled into groups of 12 samples based on band intensity from a 1.5% agarose gel image to reduce quantification and clean-up costs. Pools were subsequently cleaned with 2X Sera-Mag Speed Beads and quantified for final library pooling on a Qubit 2.0 using the dsDNA HS assay kit (Thermo Fischer Scientific, Waltham, MA). Libraries were run on both 454 machines following manufacturer's guidelines. Resulting reads were analysed with jMHC version 1.0 (Stuglik et al., 2011) to de-multiplex samples and assign genotypes.

A separate set of primers specific for *Rana arvalis* was designed and tested for Illumina MiSeq 2 × 250 bp paired-end sequencing. As Illumina sequencing generates fixed sequencing lengths and thus does not produce the subset of shorter reads found in 454 sequencing, we included the complete exon 2 and part of intron 1 in the design. Both forward (Elf_1) and reverse (Elr_2) primers were modified for the Illumina MiSeq platform with an individual 8 bp barcode and a sequence of three random nucleotides (NNN) to facilitate cluster identification (see SI; Gaigher et al., 2016). Each amplicon was marked with an individual combination of a forward and a reverse barcode for multiplexed sample identification. Every sample was amplified twice with a different barcode primer combination to avoid the assignment of false alleles in the genotyping process. PCR products were run and visualized on a 1.5% agarose gel and extracted using the MinElute Gel Extraction Kit (Qiagen. Sollentuna, Sweden). The concentration of each sample pool was measured with Quant-iT PicoGreen dsDNA assay kit (Invitrogen Life Technologies, Stockholm, Sweden) to combine pools in equimolar amounts for final library preparation. Libraries were generated using the Illumina Truseq DNA PCR-Free Sample preparation kit (Illumina Inc, San Diego, CA). Libraries were sequenced at the SNP&SEQ Technology Platform hosted at SciLifeLab in Uppsala (Sweden). Sequencing data was extracted and analysed using FLASH (Magoc and Salzberg, 2011) to combine paired reads into a single sequence and jMHC 1.0 (Stuglik et al., 2011) was subsequently used to de-multiplex samples and assign genotypes.

3. Results

3.1. Genome walking and Sanger sequencing

We generated 420 bp of novel, upstream MHC class II

sequence additional to the sequence data previously obtainable using the class II exon 2 forward primer for anurans (MhcF; Hauswaldt et al., 2007). Genome walking produced a total of four alleles among the six *L. chiricahuensis* individuals (GenBank ID KY587171-74) and three alleles from the four *L. yavapaiensis* individuals (Genbank ID KY587153-55). Using the primers designed from the genome walking sequences (Table 2; Fig. 1), we Sanger sequenced the complete exon 2 from 52 individuals across 17 species and recovered a total of 38 alleles (GenBank ID KY587141-52, KY587156-70 and KY587175-85). No more than two alleles were found per individual, indicating that these intron-based primers likely only amplify a single locus. We infer that this is the same expressed locus presented in Kiemnec-Tyburczy et al. (2010) as we are using the same reverse primer (B1intron2_R) and one of our alleles (*L. yavapaiensis* B) is identical to the expressed allele found in their cDNA sample. We were unable to align the *Nanorana parkeri* genome to our dataset because the intron sequences were too divergent. Of the four identified MHC class II loci in *Odorrana tormota* (Shu et al., 2013), our dataset could only be aligned to the highly variable Odta-A locus, as the introns were too divergent for all three other loci. The final dataset therefore included all complete exon 2 sequences, as well as *Odorrana tormota* Odta-A (GenBank ID: KC414849). Two of these 39 alleles had unique introns but identical exons, thus selection analyses were conducted using only the 38 sequences with unique exons.

3.2. Amino acid properties

Nucleotide diversity and Tajima's D were exceptionally high among PBR codons as compared to other regions of the alignment (Table 3). Previously generated cDNA sequences (Kiemnec-Tyburczy et al., 2010) and the fact that no stop codons or frame shift mutations were detected suggest that all alleles are functional and that the locus does not represent a pseudogene (Fig. 2). Amino acids were highly conserved along the 18 residues identified as conserved codons in MHC class II proteins (Kaufman et al., 1994) and only *Pelophylax lessonae* differed in more than 1 position (3/18 residues; Fig. 2). All other 38 sequences were identical across these 18 amino acids except for amino acid positions 16 and 88 of our alignment. Threonine, which is important for glycolysation, was conserved across all species at amino acid position 16 except for the two *L. virgatipes* alleles, which instead coded for methionine. At amino acid position 88, arginine was conserved in 20 of the 39 alleles and has been classified as an important amino acid in the immunoglobulin-like domain. In the remaining 19 alleles found across ten species and all four genera, a leucine occurred at position 88.

3.3. Phylogenetic reconstruction

PartitionFinder did not identify a significant partition within the exon or within the intron. The model with the lowest AICc for the complete exon was the HKY model with invariant sites and a gamma distribution (HKY + I + G), whereas the lowest AICc for the intron was the HKY model without invariant sites but with a gamma distribution (HKY + G). For both the exon and intron tree, MrBayes average standard deviation of split frequencies were below 0.01 and the Potential Scale Reduction Factors (SPRF) were at 1.00, showing that the two runs had sufficiently converged after 10 million generations. In the exon-based genealogy (Fig. 3), alleles fall into clades comprising multiple species and genera, demonstrating both trans-specific and trans-generic polymorphism relative to established taxonomy from published phylogenies (Hillis and Wilcox, 2005; Wiens et al., 2006). Several tips across the tree also exhibit long terminal branches, a common finding in MHC-based trees indicating persistence of alleles over long periods of time (Richman, 2000).

Table 3
Nucleotide diversity statistics for the alignment of 39 alleles. S = Segregating sites, ps = segregating sites out of total sites, Θ = ps/a1, π = nucleotide diversity, TajimaD = Tajima's test statistic. 'PBR overlap' are the 13 codons found in both the Brown and Tong models whereas 'PBR either' are the codons that are found by either Tong or Brown or both. 'Non-PBR either' are all 63 codons that were not found in either model.

Subset	Size (bp)	Codons	S	ps	Θ	π	TajimaD
Whole Exon	267	89	103	0.39	0.09	0.11	0.69
PBR Tong	45	15	36	0.80	0.19	0.28	1.72
PBR Brown	72	24	46	0.64	0.15	0.21	1.44
PBR either	78	26	51	0.65	0.15	0.21	1.33
PBR overlap	39	13	31	0.79	0.19	0.29	1.94
Non-PBR either	189	63	52	0.28	0.07	0.07	0.02

3.4. Co-phylogenetic analysis

Phylogenetic trees based on the mitochondrial marker as well as the intron sequences were strongly congruent and largely consistent with previously reconstructed species relationships (Fig. 4, top; Hillis and Wilcox, 2005; Wiens et al., 2009). Although there were 5 minor differences within genera between intron and mitochondrial genealogies, the overall tree structure had a moderately high Kendell coefficient of concordance (Table 4; W: intron ~ mito = 0.80). In contrast, the exon-based tree was highly incongruent with previously generated species relationships and demonstrated multiple occurrences of trans-specific and trans-generic MHC polymorphisms (Fig. 4, bottom). Several alleles were placed within different genera, resulting in a low congruence score (Table 4; W: exon ~ mito = 0.66). Interestingly, the intron versus exon co-phylogeny showed a mixed pattern (Fig. 4, middle), with strong congruence in parts of the tree and major differences in others, and had a corresponding intermediate congruence score (Table 4; W: exon ~ intron = 0.75).

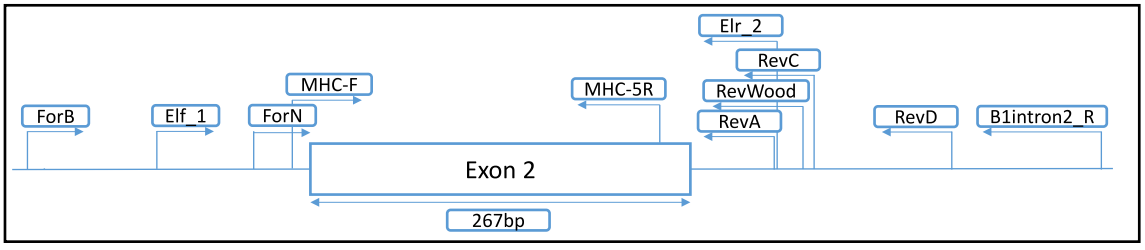

Fig. 1. Schematic diagram of primer locations along MHC class II exon 2 and flanking introns. For primer sequences see Table 2.

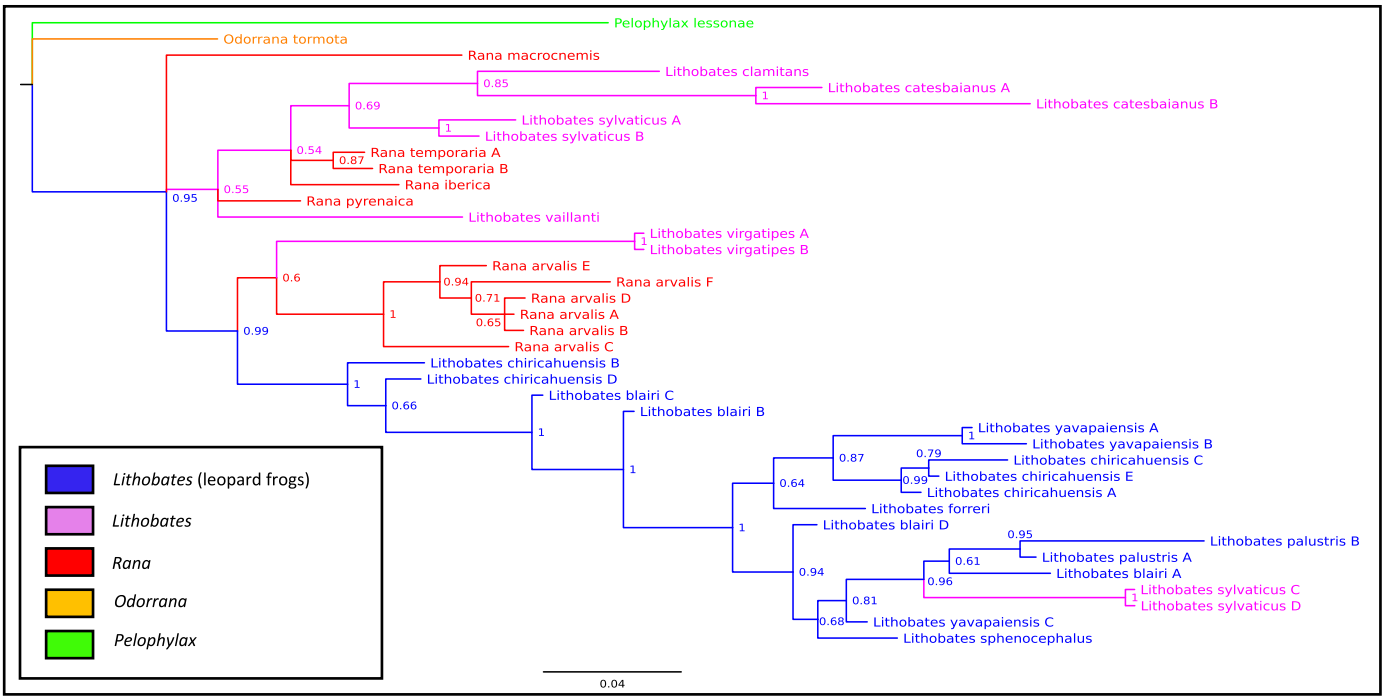

Fig. 2. Exon 2 amino acid alignment of 89 codons for all 39 recovered alleles. Black dots correspond to the consensus sequence. The top coloured bars are codons identified as present in the peptide binding region by Brown (red), Tong (yellow) or both Brown and Tong (orange) and the bottom blue bars are conserved codons as identified by Kaufmann. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

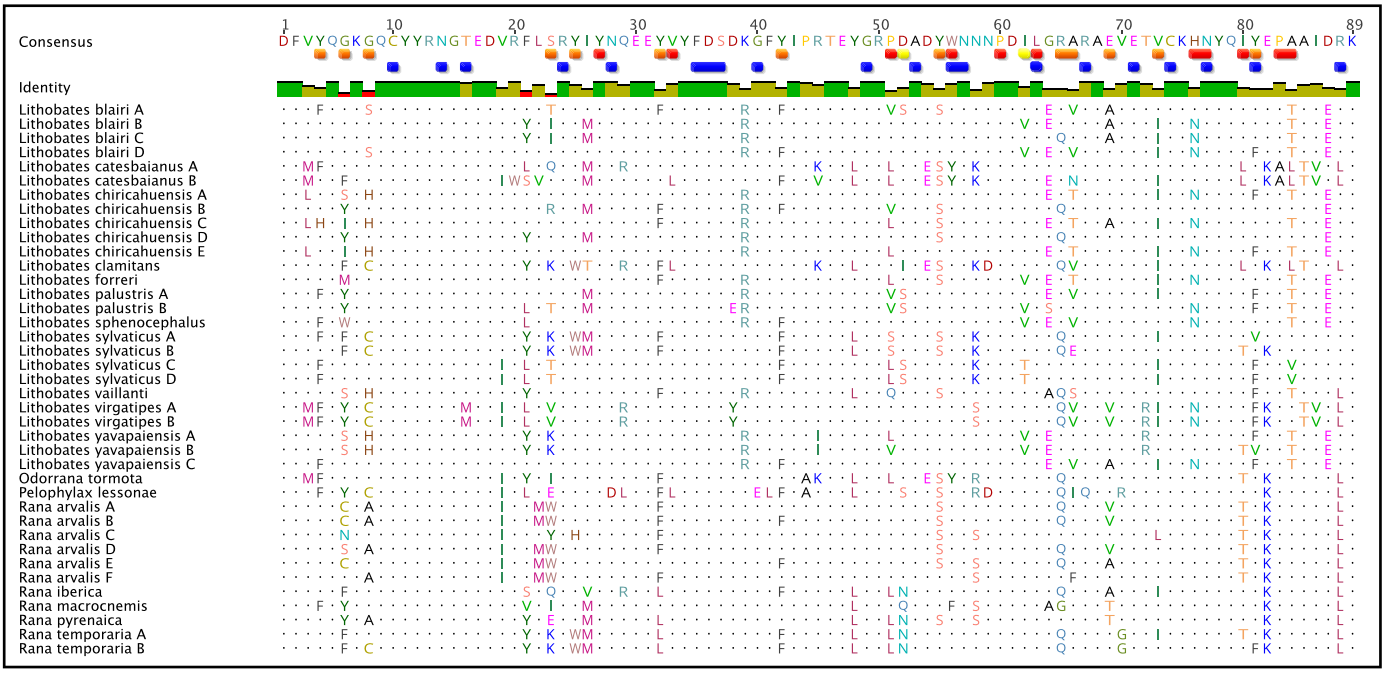

Fig. 3. Bayesian genealogy of the complete MHC class II exon for all 39 alleles. Nodes are labelled with posterior probabilities and all nodes under 0.50 are collapsed. Sequences are labelled according to genus with a separate distinction for the subgroup of leopard frogs from within the *Lithobates* genus. The identification of leopard frogs is based on the subgenus of *Pantherana* (Yuan et al., 2016).

3.5. Selection analyses

We detected a significant signature of exon-wide positive selection using PARRIS (likelihood ratio test score = 67.3; *P*-value < 0.01). The five codon-based analyses were variable in the number of negatively selected codon sites detected (2–9), but were more consistent in the number (6–10) and position of positively selected codons (Table 5). The majority of positively selected sites were also found within the PBR of both the Brown and Tong models (7/10), with two of the remaining three sites (codon 52 and codon

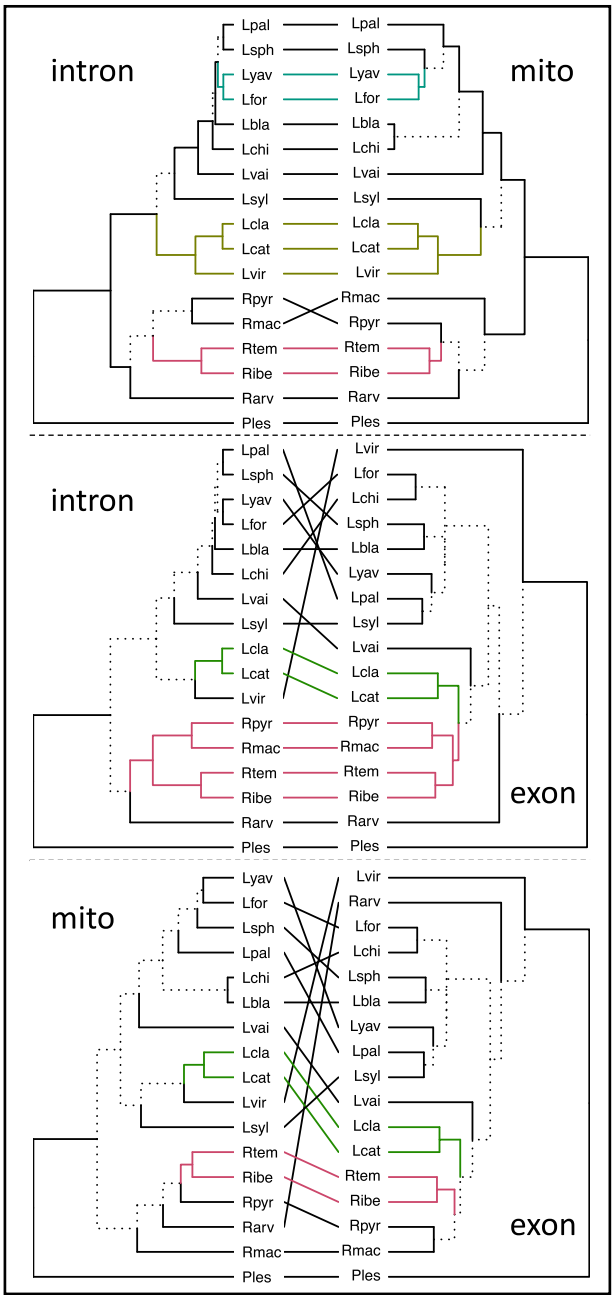

Fig. 4. Co-phylogenies of all combinations of markers. Figures were built with the Tanglegram function in the dendextend R package. Dashed branches differ between both phylogenies and coloured clades are identical in both trees. See supplemental material for the original phylogenies with support values included.

Table 4
Co-phylogenetic comparisons between three different markers. Topology and branch score differences were calculated with Phangorn and CADM values were calculated with APE, both implemented in R.

	Differences in topology	Branch score difference	CADM
intron ~ mtDNA	5	0.25	0.80
intron ~ exon	10	0.18	0.75
exon ~ mtDNA	12	0.23	0.66

84) identified as a PBR site in either the Brown or Tong model. This pattern is also reflected in the high value of Tajima's D for the PBR models (Table 3). Codon 21 was the only site found to be under positive selection (5/5 methods) that is not in the PBR as defined by either the Brown or Tong models. MEME found the same nine positively selected codons as FUBAR but additionally identified codons 25, 48, 56, 65, 70, 81 as being under positive selection.

Excluding codon 2, all sites that were found to be under negative selection by at least two methods are conserved codons across vertebrates (Kaufman et al., 1994). Codon position 74 is known to be important for di-sulfide bond formation. All others were not assigned to a specific purpose by Kaufman et al. (1994), but were found to be conserved across several distant vertebrate taxa. The three sites that were only found to be under negative selection by REL were also identified by Kaufman et al. (1994) and assigned as di-sulfide bond location (codon 10), unassigned (codon 35) and an Immunoglobulin-like codon (codon 88).

3.6. High-throughput sequencing resources

For 454 chemistry, we designed the primers ForN and RevA that amplify the entire PBR of exon 2 and only lacking the first 6 bp of the exon, which were identical in all screened species (Fig. 1 and Table 2). To multiplex samples, we generated 27 unique five bp barcodes in the ForN primer in addition to the 454 LibA adapter. A total of 42 barcoded RevA primers were also designed but nine of them were removed, as they underperformed during emulsion PCR resulting in lower coverage for these samples even when pooled at equimolar amounts (data not shown). Each of these nine primers had a C at position 5, suggesting that a particular secondary structure was the cause of poor performance, but the underlying cause could not be determined. Consequently, we generated a total of 33 adequate reverse primers to pair with the 27 forward primers (see supplemental material). When multiplexed, these barcoded fusion primers can amplify and sequence up to 891 different samples on one region of a 454 plate. Due to an indel in the RevA region for three species (*L. sylvaticus*, *L. vaillanti*, *P. lessonae*), separate reverse primers were designed for these species that could be paired with the same ForN primer. Fusion primer testing on a subset of the species revealed a high success rate but there was some inconsistency in amplification success between species (Table 1). Excluding L. *yavapaiensis*, which strongly underperformed in certain populations, average success rate was 87% on the first try (Table 1). We predict this success rate will increase if DNA extractions and PCR amplifications are repeated to the recommended three replicates.

The Illumina reverse primer location was the same as the RevA primer but including one additional base pair (a C at the 3' end) to increase the melting temperature to be more similar to the forward primer. The Illumina forward primer was located 44 bp upstream from the ForN primer, producing an amplicon of ~342 bp including the complete exon 2 and flanking regions (see Fig. 1). Taking into account barcodes and primer locations, this amplicon size allows for a 99 bp overlap of the forward and reverse reads on a 2 × 250 bp MiSeq to facilitate correct pairing of the reads. Eight forward and eight reverse barcodes were designed (see supplemental material), which is sufficient to multiplex and sequence 64 individuals per Illumina adapter combination. Performance testing revealed that 210 out of 240 individuals from 12 different populations located along a 1700 km gradient (from Hannover in northern Germany to Ludeå in northern Sweden) could be amplified and sequenced with good coverage, resulting in a total of 57 alleles (Cortazar-Chinarro, unpublished).

4. Discussion

We used genome walking to generate sequence data for the MHC class IIβ intron 1 and 5' region of exon 2 in the Ranidae.

Table 5
Positive or diversifying selection (P) and negative or purifying selection (N) across five site-specific tests of selection. Plotted are only codon positions that were found by at least two of the methods and codons identified by only one method are in the final column. Shaded in grey are codon positions that were found by both Brown and Tong to be part of the PBR. *Codon position 52 was found by Tong only and 63 and 84 by Brown only. Underlined codon positions were codons found to be conserved by Kaufman. † in REL is the Bayes factor cut off point and for FUBAR it is the false positive rate corresponding to a posterior probability of 0.984.

Method	*P*-value	Total Pos	Total Neg	2	6	8	21	23	36	37	51	52*	53	55	63*	69	73	74	84*	Additional Codons
FEL	0.05	9	6	N	P	P	P	P	N	N		P	N	P	N	P	P	N	P	
SLAC	0.05	6	3		P		P	P	N	N	P				N	P	P			
REL	100 †	9	6	N	P	P	P	P	N	N	P	P	N	P	N	P	P	N		81P, 10N, 35N, 88N
iFEL	0.05	7	3		P		P	P	N	N	P	P			N		P		P	58P
Fubar	0.05 †	9	2		P	P	P	P		N		P		P	N	P	P		P	

Combined with previously characterized intron 2 sequence information (Kiemnec-Tyburczy et al., 2010), we now have robust markers for sequencing the complete exon 2 and flanking intronic regions for the hypervariable and functionally important region of an immunogenetic marker in a widespread anuran group facing a multitude of infectious disease threats. The primers we developed, based on our novel sequence data, reliably amplify this region for at least three genera. We also demonstrated that these primers can be used with a high success rate in high-throughput multiplexed amplicon sequencing when combined with appropriate barcodes and adapters. The large number of indels and variable regions among the screened species, as well as the inability to align the introns to the closest available whole genome (*N. parkeri*, which shares a most recent common ancestor at around 85 mya; Wiens et al., 2009), shows that even with current high throughput techniques, genome walking methods are still a necessary, feasible and cost-effective alternative for developing MHC resources in a clade of interest with limited genetic resources. Intron sequences and intron-based primers also allow for the identification and subsequent sequencing of a single locus, circumventing problems associated with multi-locus sequencing and analyses (Meyer-Lucht et al., 2016).

The alignment of 39 sequences showed little variation in the conserved codons but very high variation in the PBR codons as defined by both the Brown and Tong models. The codons corresponding to the smaller Tong model (15 codons) produced a slightly higher Tajima's D value than those corresponding to the larger Brown model (24 codons). The Tajima's D value for the codons that overlapped between both models was larger than for either model alone, suggesting that codons found by both models (13 codons) are potentially the most important codons determining peptide binding in amphibians. The high number of unconserved codons for the *P. lessonae* allele might suggest it is not functional and perhaps unexpressed, but the absence of stop codons or frameshift mutations would be remarkable and unlikely if this locus was indeed a pseudogene. One possibility is that in *P. lessonae* a different class II locus plays the classical role in the immune response, allowing the locus we sequenced to be less constrained by typical codon conservation, but further MHC characterization is necessary to resolve this unusual result.

We identified a small number of amino acid substitutions that were unexpected based on conserved sites identified in previous class II studies (Kaufman et al., 1994). The polymorphic amino acid at position 16 was only present in both alleles from one *L. virgatipes* individual, where threonine was replaced by methionine. Compared to other datasets, this position is conserved across most amphibian species as a threonine (179/196 samples in Bataille et al., 2015) but two species (*Ambystoma tigrinum* and *Andrias davidianus*) had a serine (17/196 samples). Although this demonstrates that the amino acid is not entirely fixed, serine and threonine are both small, polar amino acids with a hydroxyl group (Livingstone and Barton, 1993), making this switch more likely to be stable than the sulfur-containing methionine configuration in *L. virgatipes*. However, in more distant clades (several fish species and the reptile *Amblyrhynchus cristatus* from Lillie et al., 2016), physiochemically distinct amino acids occur at this position, suggesting that this residue can be highly variable without eradicating function. In amphibians, more individuals and species must be sequenced and functional studies conducted to confirm whether this is a species-specific and stable allele change or an anomaly. A more common substitution was the leucine alternative to arginine at position 88 found in 11 species and in all screened genera of our dataset. Although these two amino acids do not share many functional properties, their frequency suggests that this alternative amino acid is another stable configuration of the MHC protein structure. As codon 88 is found in both Brown's model of the PBR but also as a conserved codon in Kaufman et al. (1994), it is likely an important codon for pathogen recognition as well as for the stable configuration of the molecule. When compared to the alleles presented in Lillie et al. (2016) the functionally similar amino acid lysine is found across teleosts, whereas the aromatic tryptophan is present in *A. davidianus*. In summary, although both codon positions 16 and 88 are more conserved across clades than expected, our comparisons with more distant clades indicate that the protein is likely also stable with (certain) different amino acid configurations.

Co-phylogenetic analyses (Fig. 4) show that the exon-based genealogy is highly incongruent compared to both the mitochondrial and the intron-based trees, which largely follow previously identified species relationships (Hillis and Wilcox, 2005; Yuan et al., 2016). This difference is also apparent from the 39 allele genealogy (Fig. 3), in which many alleles do not group by species and even the genera form polyphyletic clades. In contrast, although there is some switching between species in the intron-based tree, most samples group by species and all the genera are monophyletic, highlighting that even adjacent regions within a single locus can show distinct evolutionary histories based on different selective forces. Although located in a different part of the genome (nuclear versus mitochondrial), the intron and mitochondrial reconstructions are

comparable in topology and likely follow the actual species relationships. In contrast, branch score differences as calculated by Phangorn (Table 4) highlight larger differences in average branch lengths between the mitochondrial marker and the two nuclear markers (intron and exon). This temporal difference likely arises from differences in mutation rates between nuclear versus mitochondrial genomes that may have a stronger effect on branch lengths of the mitochondrial marker than the balancing selective forces acting on the MHC locus.

A number of evolutionary processes may explain why some loci produce genealogies that are inconsistent with known species relationships, and this trans-species polymorphism (TSP) is known to occur at especially high rates in coding regions of immune system genes (Těšický and Vinkler, 2015). Convergent evolution and incomplete lineage sorting are the two main mechanisms in which two closely related alleles are found in distantly related taxa, and both phenomena can occur more frequently with higher selection pressures. For example, positive selection mediated by similar pathogens could lead to convergent evolution of functionally comparable MHC alleles in different species (Kriener et al., 2000; Srithayakumar et al., 2012). Balancing or frequency-dependent selection can also maintain the same alleles in divergent species for longer than would be expected if they were neutral and undergoing genetic drift (Ottová et al., 2005; Těšický and Vinkler, 2015). At present, we cannot distinguish between the two forces in our dataset as more information on parasite regimes and genome-wide evolutionary history is needed (Lenz et al., 2013). However, clades where the intron and exon show more convergence than with the mitochondrial marker are likely the result of incomplete lineage sorting caused by balancing selection (e.g., the genus *Rana*), whereas strongly divergent intron and exon phylogenies are more likely to be due to positive selection acting on the exon, resulting in convergent evolution across different species (e.g., *Lithobates*).

Strong exon-wide positive selection was detected on the exon 2 using the PARRIS method and this was concentrated in certain codons as evident from the codon-based selection analyses (Table 5). Although each analysis produced some unique results, there was a large overlap in the codon positions identified as being under selection across the five methods. The majority of differences relate to how conservative a method is in identifying codons under selection; for example SLAC (9 total codons) is more conservative than Bayesian empirical approaches like REL (19 total codons; Kosakovsky Pond and Frost, 2005a). One important caveat is that most methods should detect the same codons in large datasets, but may show stronger differences with small sample sizes (Kosakovsky Pond and Frost, 2005b). Because the internal fixed effects likelihood method (iFEL) detects selection on the internal branches and the other methods focus on terminal branches (Kosakovsky Pond et al., 2006b), iFEL reflects older selective pressures relative to other codon-based analyses. In light of this distinction, positive selection detected at sites 8, 55 and 69 based on at least 3 methods, but not detected using iFEL, are likely experiencing positive selection from more recent pressures such as emergent infectious pathogens.

The majority of codons under positive selection in this study are part of the PBR as defined by the Tong and Brown models, consistent with evolutionary theory that predicts that pathogen-mediated selection will mostly act on the amino acids important for antigen-recognition (Hughes and Hughes, 1995). There was also strong overlap with important survival-associated PBR codons as found in experimental Bd infection studies; 1/1 sites overlapped with findings from Savage and Zamudio (2011) and 3/5 sites overlapped with results from Bataille et al. (2013). Codon 21 was identified by all five methods but is not part of either the Brown or Tong model. This codon has previously been identified as under positive selection in several other subsets of amphibians (Bataille et al., 2015; Kiemnec-Tyburczy et al., 2010; Lillie et al., 2015). Although there was overlap in some of the species used in these different datasets, the consistency of finding this codon in several clades and publications with different methods shows that it might be an important codon specifically for amphibian pathogen recognition. Normalized dN/dS values of a mixed vertebrate dataset (Lillie et al., 2016) show that codon 21 has a higher value for the 23 analysed amphibians (0.38) compared to the other 32 vertebrates (−2.13), again suggesting that there is perhaps a stronger pattern of positive selection for amphibians at this site.

All but one of the five negatively selected codons we identified (codon 2) were previously identified by Kaufman et al. (1994) as vertebrate-wide conserved codons. Although the remaining four were classified as unassigned structural codons, the overlap with previous vertebrate-wide analyses confirms that these are important amino acids for the correct structure and function of the protein. Codon 2, coding for phenylalanine, was not previously found to be particularly conserved (Kaufman et al., 1994) and therefore this may be an amphibian-specific conserved codon. Across the 125 amphibian sequences used in Bataille et al. (2015), 68 were also phenylalanine and 67 coded for tyrosine, which is a similar aromatic amino acid and thus likely maintains a conserved function. Taken together, the strong evidence of negative selection on functionally important amino acids and positive selection on the amino-acids in the PBR across all screened species are indicative of a functionally expressed MHC locus that is under strong selective pressures.

Both 454 and Illumina adapters successfully amplified a single locus in the majority of samples (Table 1), confirming that these primer sets can be used for population genetic purposes. Although MiSeq Illumina technology is cheaper on a per sample basis, both Illumina and 454 sequencing technologies are viable options for multiplexed MHC genotyping in frogs of the Ranidae. Although 454 technology has been discontinued, the overlap between 454 and Ion Torrent technology suggests that our adapter design here should be robust for use on this similar platform. Regardless of the choice of platform based on cost and read quality, both of which are continually improving, the multiplex-ready adapter design and development presented here is an important genetic resource enabling parallel sequencing of hundreds of individuals with limited cost and a fast turnaround time for a multitude of species (Dheilly et al., 2014). Future studies of the MHC in the Ranidae can reduce data generation efforts by using these sequences and primer sets as a launching point, avoiding the considerable time and effort required to develop species-specific intronic resources and subsequent fusion primer design and optimization. Our fusion primers were designed specifically to stay within current sequencing technology limits and to target a single locus, therefore minimal optimization should be needed to go from DNA extraction to genotyping. Allelic dropout is always an issue, especially for highly variable markers like the MHC, and a frequent problem for non-model organisms without genome-wide information. We are likely missing a small fraction of total alleles given the number of species and individuals we sequenced, but this is true for any primer-based sequencing of MHC loci, and both high coverage and PCR repetitions should minimize these issues. Although in one species (*L. chiricahuensis*) we only recovered a total of five alleles (Table 1), low MHC diversity in this species does not suggest severe allele drop out. Because this species is the only Federally threatened frog included in our study and has declined rapidly throughout its range (Sredl and Jennings, 2005), it is more likely that reduced MHC diversity is the result of population bottlenecks (Mainguy et al., 2007). Although only 17 species were tested, these three genera

contain a total of 114 species, of which 33% are considered threatened (IUCN, 2016). Given the impact that Bd has had on amphibians worldwide, and given that these genera contain Bd susceptible, resistant and tolerant species, these resources can provide much needed immunogenetic information and help shed light into pathogen dynamics. These markers can additionally be used for other evolutionary studies of adaptation, mate preferences, and as a marker for monitoring population genetic diversity.

5. Conclusions

The presented use of genome walking and intron-based primers are a blueprint for developing single-locus amplicon sequences for new clades of interest that lack a reference genome. The newly developed primers for the Ranidae class II MHC will provide an important resource for future studies on immunogenetic variation in frogs, especially given the many disease threats amphibians are currently facing. The distant primers up- and downstream of the exon (ForB and RevL) provided the most reliable amplification success across all 3 genera and are a good starting point to characterize the full exon and adjacent introns in other related species. Their fragments lengths make them unsuitable for current high-throughput sequencing (HTS) methods but they form a basis for clade-specific primer design closer to the exon-intron boundaries. Two different primer sets developed for HTS successfully amplified both subclades of the Ranidae and were compatible with both 454 as well as Illumina sequencing using barcoded fusion primers to multiplex hundreds of samples simultaneously. For 17 species of Ranidae spread over 3 genera, both the exon and intron showed high levels of variation and there were strong signals of both positive as well as negative selection acting across the exon. All positively selected codons were found to be within the PBR, except for codon 21 which is likely an amphibian specific important codon. Phylogenetic analyses between intron, exon and a mitochondrial marker showed strong signs of trans-species polymorphism for the exon and highlight the distinctive selective pressures acting on the different loci. These sequences and primers will provide a useful genetic resource for future studies of MHC evolution in Ranid frogs.

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Author contributions

AES and KPM conceived the study design. AES, EHCG, AC, DJH, MC provided samples. AES, DJH and RCF provided funds and laboratory space. KPM and MC performed all the labwork and KPM and AES analysed the data. KPM and AES wrote the manuscript with input from all other authors.

Competing interests

The authors have no conflicts of interest or competing interests to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2017.05.022.

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