Conserv Genet
DOI 10.1007/s10592-017-1001-3

RESEARCH ARTICLE

Lost but not forgotten: MHC genotypes predict overwinter survival despite depauperate MHC diversity in a declining frog

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Received: 24 February 2017 / Accepted: 27 July 2017

Abstract The amphibian disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has contributed to the decline of Chiricahua leopard frogs (*Rana chiricahuensis*), a federally threatened species native to the Southwestern United States. We characterized immunogenetic variability in *R. chiricahuensis* by sequencing an expressed Major Histocompatibility Complex (MHC) class IIβ gene across 13 natural populations in Arizona, USA, as well as 283 individuals that were captive reared from two egg masses. We recovered a total of five class IIβ MHC alleles compared to 84 alleles previously characterized in eight natural populations of the Arizona congener *R. yavapaiensis*, demonstrating reduced MHC diversity in *R. chiricahuensis*. One allele was fixed in five populations but none of the *R. chiricahuensis* alleles were closely related to *R. yavapaiensis* allele Q, which is significantly associated with chytridiomycosis resistance in laboratory trials. Nine of 13 *R. chiricahuensis* population localities were *Bd* positive, and bearing allele RachDRB*04 was the best genetic predictor of an individual being infected with *Bd*. A total of three class IIβ alleles were recovered from captive reared individuals, which were released to two natural population localities followed by recapture surveys to assess MHC-based survival over winter, the time when chytridiomycosis outbreaks are most severe. At one site, all released animals were fixed for a single allele and MHC-based survival could not be assessed. At the second site, fewer than half of the released but all of the recaptured individuals were homozygous for RachDRB*05, indicating that MHC genotype is important in determining *Bd* survival under natural field conditions. We conclude that the limited MHC variation in *R. chiricahuensis* is likely the consequence rather than the cause of natural selection favoring alleles that promote survival in the face of *Bd*. Our study highlights that preserving even low levels of functional genetic variation may be essential for population persistence, and that local disease adaptation may present as a reduction in genetic diversity. These finding also suggest that for populations that have declined due to a specific infectious pathogen, MHC-based genetically-informed reintroduction approaches may enhance species recovery efforts.

Keywords Peptide binding region · *Rana* · Chytridiomycosis · *Bd*

Electronic supplementary material The online version of this article (doi:10.1007/s10592-017-1001-3) contains supplementary material, which is available to authorized users.

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Introduction

Maintaining genetic diversity is critical for the persistence of natural populations (Lande 1988). Consequences of reduced genetic variation include reduced survival, diminished reproductive output, slower growth rates, and an inability to adapt to environmental change (Lacy 1997;

Frankham and Ralls 1998; Reed and Frankham 2003; Mattila and Seeley 2007; Reid et al. 2016). Genetic diversity can be particularly important for preventing infectious disease outbreaks and epidemics (Waldman and Tocher 1998; Coltman et al. 1999; Meagher 1999; Cassinello et al. 2001; Little 2002; Altizer et al. 2003; Spielman et al. 2004; Simone-Finstrom et al. 2016). Because the Major Histocompatibility Complex (MHC) comprises numerous polymorphic immune-related genes that are conserved across vertebrate animals (Flajnik and Kasahara 2001), MHC genes are common targets of wildlife studies assessing whether functional genetic variation has been depleted from natural populations, especially when pathogens are suspected as the cause of population declines (reviewed in Bernatchez and Landry 2003; Trowsdale 2011). The MHC encodes cell-surface glycoproteins that bind pathogen molecules and present them to T-cells to initiate acquired (or T-cell dependent) immunity (Germain 1994). Class I MHC molecules primarily recognize intracellular pathogens (i.e., viruses), whereas class II molecules bind to extracellular pathogens (bacteria and fungi); for both classes, the majority of DNA variation resides in the Peptide Binding Region (PBR) that is responsible for binding to pathogen molecules (Hedrick and Kim 1999). This central role in initiating and regulating the immune response creates strong selection on MHC loci for numerous polymorphisms and gene copies, thereby maximizing the array of pathogens that can be recognized (Simpson 1988). A lack of MHC variation is thus a useful biomarker for detecting a severe loss in overall functional population genetic diversity.

Natural populations often harbor large numbers of MHC alleles per functional locus due to balancing selection caused by pathogens. Negative frequency-dependent selection, wherein pathogens rapidly adapt to common MHC genotypes and enable individuals with rare MHC genotypes to have a fitness advantage, is one mechanism that favors extreme MHC diversity within populations (Borghans et al. 2004; Eizaguirre et al. 2012). Heterozygote advantage and copy number variation is an alternate mechanism for pathogen resistance, because MHC heterozygotes have twice as many PBR conformations and can thus bind to a wider array of pathogen molecules (Doherty and Zinkernagel 1975; Hughes and Nei 1992). However, natural wildlife populations that have undergone extreme population bottlenecks show reduced or absent MHC diversity, including examples from mammals (O'Brien et al. 1985; Babik et al. 2005; Ellegren et al. 1993; Radwan et al. 2007; Zhu et al. 2007; Mainguy et al. 2007), birds (Hansson and Richardson 2005; Bollmer et al. 2007; Sutton et al. 2015), and amphibians (Babik et al. 2008; Höglund et al. 2015). These examples of low MHC diversity are considered extreme because balancing selection can maintain MHC polymorphism even when the effects of drift are strong (Hedrick 2002; Sutton et al. 2011). However, the decline of MHC diversity may be accelerated if selection continues to act on allele frequencies following a bottleneck, because ongoing selection for pathogen-resistant genotypes may cause the optimal MHC allele to fix at a rate far higher than the loss of rare alleles due to drift (Ejsmond and Radwan 2011; Sutton et al. 2011). Empirical evidence exists for both maintenance (Oliver and Piertney 2012; Wenink et al. 1998) and accelerated loss (Eimes et al. 2011) of MHC diversity following a bottleneck, and more studies in natural systems are needed to understand the selective landscape that causes MHC variation to be lost or maintained.

In amphibians, the most notorious and devastating pathogen characterized to date is *Batrachochytrium dendrobatidis* (*Bd*), a chytrid fungus that causes the skin disease chytridiomycosis (Berger et al. 1998). *Bd* is linked to amphibian mass mortality events and declines at locations across the globe (Stuart et al. 2004; Berger et al. 2016), including the southwestern United States (Bradley et al. 2002; Savage et al. 2011), although in many other regions endemic *Bd* lineages infect amphibians without causing noticeable declines (Becker et al. 2017). In the 1980s and early 1990s, mass mortality events were observed in numerous Arizona and New Mexico anuran populations, but were attributed to "postmetamorphic death syndrome" (Scott 1993) as *Bd* had not yet been identified. Subsequent analyses in the southwestern U.S. have detected *Bd* in museum specimens of the Tarahumara frog (*Rana tarahumarae*, now extirpated in the U.S.) and lowland leopard frog (*R. yavapaiensis*) dating back to 1972 (Hale et al. 2005). *Bd* has also been linked to ongoing ranid frog die-offs in natural Arizona populations (Bradley et al. 2002; Savage et al. 2011, 2015), and controlled lab infections have induced fatal chytridiomycosis in Arizona *R. yavapaiensis* individuals (Savage and Zamudio 2011, 2016). However, the southwestern amphibian that has declined most severely from chytridiomycosis is the Chiricahua leopard frog (*Rana chiricahuensis*; Sredl and Jennings 2005). Currently, *R. chiricahuensis* is federally listed as Threatened, and chytridiomycosis has been associated with numerous population extirpations and die-offs in *R. chiricahuensis* (USFWS 2007). In *R. yavapaiensis* populations occurring in close proximity to *R. chiricahuensis*, class IIβ MHC variation predicts susceptibility to chytridiomycosis, both within and among populations (Savage and Zamudio 2011, 2016). However, immunogenetic correlates of chytridiomycosis susceptibility have not yet been explored in *R. chiricahuensis*.

Here, we conduct the first analysis of class IIβ MHC variation in *R. chiricahuensis* to assess whether reduced immunogenetic diversity is a cause and/or a consequence of population declines in the face of chytridiomycosis. We (1) characterize PBR diversity across natural Arizona

populations, (2) compare PBR polymorphism and similarity of allelic lineages between *R. chiricahuensis* and *R. yavapaiensis*, (3) assess current and historical positive selection acting on PBR alleles, and (4) test for associations between Bd infection and PBR variation. Because chytridiomycosis outbreaks occur only during cooler winter months in the southwest (Savage et al. 2011), overwinter survival is a key metric of disease resistance. Thus, we also (5) perform mark-recapture surveys and individual genotyping on two cohorts of captive-reared *R. chiricahuensis* individuals over their first winter post release to determine whether certain PBR genotypes confer higher overwinter survival.

Materials and methods

Natural population sampling and head starting

Toe or tail clips were collected from adult and larval *R. chiricahuensis* individuals sampled from natural Arizona populations. All U.S. *R. chiricahuensis* populations have been grouped into eight Recovery Units (RUs), which are natural geographic regions defined as units where frog metapopulation dynamics function or could function as the species recovers (USFWS 2007). Tissue samples were collected opportunistically across seasons from 2009 to 2014 in RUs 1, 2, 3, 4, 5 and 7 by Arizona Game and Fish Department biologists and stored in ethanol prior to DNA extraction.

In 2013, two *R. chiricahuensis* egg masses were selected by the Arizona Game and Fish Department for head-starting (i.e., captive rearing followed by release back to the wild). The first egg mass was collected from a natural population in a southern Arizona location known as Gardner Canyon (AR), which is within Recovery Unit 2 in the *R. chiricahuensis* recovery plan (USFWS 2007). The second egg mass was collected from a natural population in a northern Arizona location known as Cabin Draw (CD), which is within Recovery Unit 5. Animals were reared at the Johnson Center head-starting lab located within the Conservation and Science Department of the Phoenix Zoo. The head-starting protocol was developed and refined for *R. chiricahuensis* management (Wells et al. 2001). Hatched larvae were distributed and reared among nine 76 L tanks, each filled with 57 liters of water and maintained at a population density of 4.4 larvae per liter. Diet consisted of freeze-thawed romaine lettuce, tadpole gel (a gelatinous dietary supplement with macro- and micronutrients), and egg whites. As larvae metamorphosed, they were moved to a metamorph container that consisted of shallow 3×1 m tanks filled with ~8 cm of water and supplied with filtering floss, haul out areas, plants and cricket feeders. After rearing through metamorphosis and prior to their release back to the wild, we collected toe-clips from the third digit of the left front leg and preserved them in 95% ethanol. The purpose of toe-clipping was two-fold: to identify frogs as head-started versus resident individuals following release back to the wild, and to genotype each head-started individual (see below). All individuals were tested for *Bd* and confirmed to be pathogen free prior to release (see below for methods).

Mark-recapture study

Toe-clipped metamorphs from AR and CD were released to sites within the RUs they were originally collected from in 2013. We selected small, relatively isolated ponds as release sites to minimize dispersal and maximize our ability to survey the entire site. We used nighttime visual encounter surveys and toe-clipping of all recaptured head-started frogs to assess genotype-based survival of released frogs over the winter of 2013–2014. Recaptured head-started frogs were toe-clipped on a different digit than the initial mark so that we could infer how many times each frog had been captured across all survey dates. We also toe-clipped any encountered resident frogs (i.e., naturally occurring at the release site, but not members of the toe-clipped and released cohort) in order to infer recapture rates for residents as compared to head-started individuals. Resident frogs were all toe-clipped on a different digit from head-started frogs so that recaptures on future survey dates could always be distinguished as a resident versus a released frog recapture. Surveys took place once per month per site in the months of October 2013, March 2014, April 2014 and June 2014 and were all conducted by the same person following a standardized survey protocol to ensure consistent sampling effort. Surveys did not take place during the months of November–February as cold winter conditions made it extremely unlikely that any animals would be observed during this time. Perimeter scans were conducted at the beginning of each survey to determine frog presence and location prior to disturbing the site, then attempts were made to capture and toe-clip all observed individuals.

Pathogen screening and MHC genotyping

To genotype frogs sampled from natural populations and head-started frogs, we extracted DNA from toe clips using DNeasy blood and tissue kits (Qiagen), eluting into a volume of 200 µL for each sample. For head-started frogs, we extracted DNA twice; once from toe-clips collected from all individuals prior to release, and again from the subset of individuals that were recaptured and re-toe clipped during surveys. The number of *Bd* Genome Equivalents (GE) per tissue sample, a measure

of infection intensity, was determined using a Taqman quantitative PCR assay designed for *Bd* quantification (Boyle et al. 2004). All samples were run in duplicate; in rare cases (<3% of samples) where replicate runs showed inconsistencies in infection status (infected versus uninfected) or at least one order of magnitude difference in infection intensity, a third replicate was run and values from the two most similar replicates were averaged.

We also used toe clip DNA to amplify exon 2 of an expressed MHC class IIβ gene known to affect *Bd* susceptibility (Savage and Zamudio 2011, 2016; Bataille et al. 2015). We used previously designed and optimized locus-specific primers (Kiemnec-Tyburczy et al. 2010; Mulder et al. 2017, under review) modified for pyrosequencing by designing fusion primers that included a 5 bp barcode on both the forward and reverse primer as well as the 454-specific adapters suitable for amplicon sequencing (lib-A). Two repetitions were performed with the ForN forward primer and one repetition was amplified with the original MhcF primer (located 3′ of ForN) to facilitate comparison with the original *R. yavapaiensis* samples and reduce the chance of primer-specific null alleles. Barcodes were designed excluding 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 even one PCR or sequencing error per barcode would not alter accurate assignment of reads to samples (Faircloth and Glenn 2012). DNA extracts were amplified using fusion primers with a unique combination of forward and reverse barcodes. All samples were run in triplicate with each replicate having a unique barcode combination to control for any potential PCR bias. Samples as well as replicates were randomized across PCRs, plate-locations as well as sequencing runs and a PCR negative was included for every PCR master mix. Reverse primers were diluted to facilitate addition of small volumes to all PCR reactions and a unique negative primer was added to each well individually by multichannel. PCRs were run for 40 cycles of 95, 54, 72 °C (each for 45 s) preceded by 5 min of 95 °C and with 10 min of 72 °C for the final extension. PCR products were run on a 1.5% agarose gel with GelRed (Biotium) and samples were pooled in groups of 12 based on their band intensity. Empty bands and 10 µL of all PCR negatives were also included in the pools. Gel-based pooling was done to reduce quantification and PCR clean-up costs, and in test runs was shown to adequately pool samples at similar molarities. The pools of 12 samples 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 four 454 junior runs and as part of a 454 XL run.

Resulting reads were analysed with jMHC version 1.0 (Stuglik et al. 2011) to de-multiplex samples and assign genotypes. We required a minimum depth of coverage of 40×, as well as at least three independent PCRs to identify a unique allele. Individuals were considered successfully genotyped if at least two of the replicates resulted in a minimum of 10× coverage when adding up both allele-counts. Individuals were inferred to be heterozygotes if at least two of the replicates had a minimum sequence read allele frequency of 0.10. Putative alleles were named according to the nomenclature rules defined in Klein et al. (1990).

Genealogical reconstruction

We aligned the *R. chiricahuensis* PBR alleles generated in this study with previously characterized *R. yavapaiensis* PBR alleles (GenBank IDs: JN638850–JN638882; Savage and Zamudio 2016) as well as the Mesobatrachian frog *Xenopus laevis* PBR (GenBank ID: D50039) included as the outgroup. The final alignment included all except the first two exon 2 codons (but not the flanking intronic sequence recovered by our fusion primers) and was generated using MUSCLE with 8 iterations implemented in Geneious 9.1 (Kearse et al. 2012) followed by manual adjustment. We used PartitionFinder 1.1 (Lanfear et al. 2012) to identify the best substitution model based on the corrected Akaike Information Criterion (AICc; Hurvich and Tsai 1993), only allowing substitution models available in MrBayes. Genealogies were then reconstructed using MrBayes 3.2 (Ronquist et al. 2012) run for ten million generations with a sampling frequency of 500 generations and excluding a burn in of 25%.

Spatial genetic analysis

We estimated the population differentiation metric *D* (Jost 2008) among populations of *R. chiricahuensis* and *R. yavapaiensis* based on PBR genotypes using SMOGD (Crawford 2010). We tested for relationships between genetic divergence and geographic distance among sample populations by performing Mantel tests with 1000 bootstrap replicates using the Isolation by Distance Web Service (Jensen et al. 2005). We used the residuals from the reduced major axis regression to create a genetic divergence landscape using the Genetic Landscapes GIS Toolbox (Perry et al. 2011a, b) in ArcGIS 10.2 (ESRI 2011). Residuals were used to remove the effects of geographic distance on genetic divergence to show areas of exceptionally high or low genetic divergence (Perry et al. 2011a, b). The residuals were mapped at the midpoints between sampling locations and an inverse distance weighted interpolation was performed to generate a continuous surface from the midpoints.

Selection analyses

We used Hypothesis testing using Phylogeny (HyPhy; Pond et al. 2005) to test for signatures of selection acting on PBR codons across the entire *R. chiricahuensis* and *R. yavapaiensis* genealogy. Prior to performing selection analyses, we tested for evidence of recombination using genetic algorithms for recombination detection (GARD) and single breakpoint recombination (SBR; Kosakovsky Pond et al. 2006b). We ran the five maximum likelihood codon-based tests of selection, including fixed effects likelihood (FEL), internal-FEL (iFEL), Fast Unconstrained Bayesian AppRoximation (FUBAR), random effects likelihood (REL), and single likelihood ancestry counting (SLAC). Where appropriate, significance was set at a p-value of 0.05. We only report significant evidence of positive selection when positive selection was detected for a branch and site using at least four of the five methods.

PBR supertyping

To collapse MHC alleles into functional supertypes based on physiochemical amino acid properties, we created an alignment including only the 13 codon positions that are known to affect peptide-binding capabilities of human class IIβ alleles (Brown et al. 1993; Jones et al. 2006). We then characterized each codon based on the five physiochemical descriptor variables that are available for all amino acids in Jombart et al. (2010): z1 (hydrophobicity), z2 (steric bulk), z3 (polarity), z4 and z5 (electronic effects) We input a matrix of these physiochemical descriptors across all 13 codons and all *R. chiricahuensis* and *R. yavapaiensis* alleles to define functional PBR clusters using discriminant analysis of principle components (DAPC) implemented in the R package adegenet 1.4-0 (Jombart and Ahmed 2011). The optimal number of clusters was determined using a change in the Bayesian Information Criterion (ΔBIC) value ≤2. All alleles falling within the same cluster were collapsed into a single PBR supertype.

Mitochondrial haplotype sequencing

We sequenced 16S and 18S rRNA subunits from one arbitrarily selected individual per population from each *R. chiricahuensis* and *R. yavapaiensis* population included in this study. To more evenly represent overall neutral genetic diversity between species, we also sequenced 16S and 18S from one individual in each of four additional *R. yavapaiensis* populations sampled in a previous study (Savage et al. 2015) and two additional *R. chiricahuensis* populations that did not reliably amplify for the PBR locus. Genomic DNA from each individual was sheared by sonication to an average size of 400 bps and prepared for Illumina sequencing using Nextera-style adapters with 8 bp barcodes. Samples were quantified using the Qubit and pooled together in pools of eight and subsequently hybridized to MYbaits probes (MYcroarray, Ann Harbor, USA) that were designed to cover the complete 12S and 18S genes of the mitochondrial genome. Libraries were sequenced on part of a single lane of a paired-end 2×150 bp Illumina HiSeq 2500. Sequences were demultiplexed and the adapters were removed using cutadapt 1.12 (Martin 2011). Reads were mapped against the reference mitochondrial genome with bowtie2.2.9 (Langmead and Salzberg 2012), using default parameters. PCR duplicates were removed with Picard 2.8.2 and consensus sequences were called requiring a minimum coverage of six reads. Consensus sequences were subsequently aligned using MUSCLE as implemented in Geneious 9.1.7 (Kearse et al. 2012) for a maximum of 16 iterations. Haplotype networks were built using TCS 1.21 (Clement et al. 2000) and edited using tcsBU (Múrias Dos Santos et al. 2015).

Statistical analyses

We measured *Bd* infection prevalence as the proportion of frogs infected out of the total number sampled per population. *Bd* infection intensity was measured as the average qPCR-inferred quantity of *Bd* present per individual among infected individuals only within each population. Significant differences in *Bd* infection prevalence across populations and sampling months were inferred using Fisher exact tests. Significant differences in *Bd* infection intensity among populations and sampling months and significant differences in population genetic differentiation within and among species were estimated using Kruskal–Wallace H tests. Multivariable logistic regression was performed to investigate the influence of each MHC allele and MHC heterozygosity on the presence or absence of *Bd* infection among *R. chiricahuensis* individuals. All models including interaction terms were compared using the Akaike information criteria (AIC) and the final model was selected using backwards selection. The significance of each variable was checked by the Wald test statistic. Finally, significant differences in recapture rates among released MHC genotypes were inferred using Fisher exact tests.

All statistical analyses were performed in R v. 3.2.5 (R Development Core Team 2008). Genetic diversity statistics for MHC and mitochondrial sequences recovered from each species were calculated using the R package PopGenome (Pfeifer et al. 2014). Multivariable logistic regression was performed using the loglm function in the R package MASS (Venables and Ripley 2002). Fisher exact tests were performed using the fisher.test function and Kruskal–Wallace tests were performed using the kruskal.test function.

We estimated the total population size, N, using Krebs' (1989) unbiased equation:

$$N = \left[\frac{(M+1)(C+1)}{(R+1)}\right] - 1$$

where M number of individuals marked initially, C total number of individuals in second sample, and R number of marked recaptures.

We calculated the 95% confidence interval as N ± (1.96) (SE), with standard error (SE) calculated as:

$$SE = \sqrt{\frac{(M+1)(C+1)(M-R)(C-R)}{(R+1)^2(R+2)}}$$

Results

We recovered a total of five MHC class IIβ peptide-binding region (PBR) alleles from 182 *R. chiricahuensis* individuals sampled from 13 natural populations (Fig. 1; Table 1; Online Resource 1; GenBank IDs MF537004–MF537008). We also extracted DNA from two additional population samples, but due to lack of reliable PBR amplification these populations were only included in the haplotype analysis (see below). In contrast to the five PBR alleles recovered in *R. chiricahuensis*, 84 PBR alleles were previously recovered from 128 individuals sampled from eight natural populations of *R. yavapaiensis* (Savage et al. 2016; Fig. 1). In *R. chiricahuensis*, RachDRB*01 was the most common of the five alleles, followed in descending order by RachDRB*05, RachDRB*04, RachDRB*03, and RachDRB*02 (Table 1). Five populations were fixed for RachDRB*01, one population was fixed for RachDRB*02, another was fixed for RachDRB*04, and the remaining six populations were polymorphic for the same two alleles, RachDRB*03 and RachDRB*05 (Table 1).

Bd infection prevalence and intensity was measured for 245 *R. chiricahuensis* individuals sampled from 13 natural populations (Table 1; Fig. 2). Mean variation among

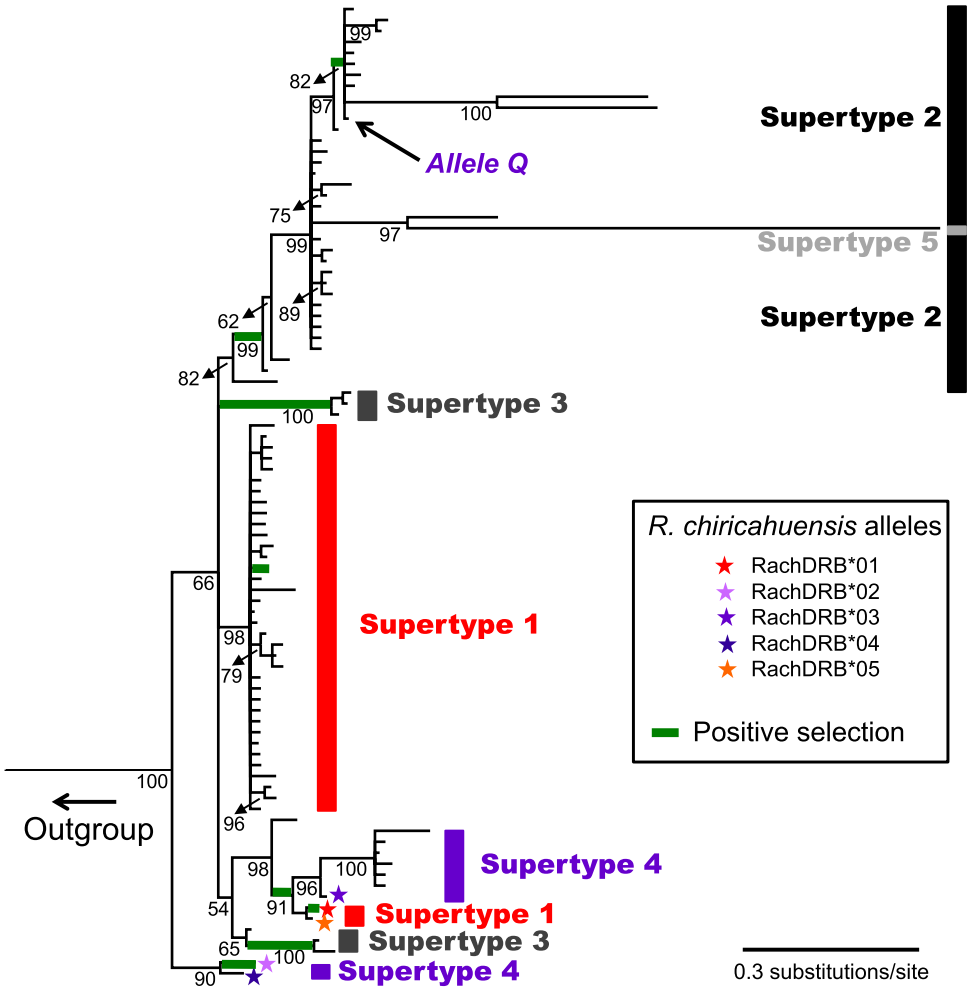

Fig. 1 Bayesian genealogy of *R. yavapaiensis* and *R. chiricahuensis* MHC class II PBR alleles with *Xenopus laevis* class II PBR (GenBank ID: D50039) used as the out group. The five *R. chiricahuensis* alleles are marked with stars; all other alleles were recovered from *R. yavapaiensis*. Branches with significant evidence of positive selection acting on codon position 46 are shown in *green*. *Horizontal bars* to the right show functional supertypes 1 (*red*), 2 (*black*), 3 (*dark gray*), 4 (*purple*) and 5 (*light gray*), which are distributed across clades. (Color figure online)

Table 1 *Rana chiricahuensis Bd* and MHC sampling in Arizona, USA Bd infection was diagnosed using quantitative PCR with a Bd-specific fluorescent probe on DNA extracted from toe clips, unless otherwise noted

Population	MHC alleles (frequency)	Month(s) sampled	No. adults (larvae)	No. Bd positive	Bd prevalence	Avg. infect. intensity[a]
Gardner Canyon (AR)	RachDRB*01(1.0)	Feb 2013	16 (10)	1/6	0.17	177
		Aug 2012	11	0/11	0.00	–
Beatty's guest ranch (BGR)	RachDRB*02 (1.0)	Apr 2012	5	0/5	0.00	–
Crouch Creek (CRC)	RachDRB*03 (0.08)/ RachDRB*05 (0.92)	Jul 2009-2011	32 (21)	0/11	0.00	–
Cabin Draw (CD)	RachDRB*03 (0.11)/ RachDRB*05 (0.89)	Unknown	9	1/1	0.11	315
Crazy Lazy P Tank (CLPT)	RachDRB*01 (1.0)	Aug 2013	10	0/10	0.00	–
Empire Spring (ES)	RachDRB*01 (1.0)	Aug 2012	31	13/31	0.42	3905
Middle March Canyon (MM)	RachDRB*04 (1.0)	March 2013	15	3/15	0.20	47
Three Forks Tank (TFT)	RachDRB*03 (0.76)/ RachDRB*05 (0.24)	Unknown	24	2/24	0.08	416
Southwestern research station (SWRS)	RachDRB*01 (0.37)/ RachDRB*04 (0.63)	Oct 2011	8	2/8	0.25	19,867
Sycamore Canyon (SY)	RachDRB*04 (1.0)	May 2013	11	2/11	0.18	63,885
West tank (WT)	RachDRB*01 (1.0)	July 2013	10	0/10	0.00	–
Upper moore (UM)[b]	RachDRB*03 (0.20)/ RachDRB*05 (0.80)	Oct 2013	7	5/7	0.71	1,003,200
		Mar/Apr 2014	9[c]	0/9	0.00	–
Bowman tank (BT)[b]	RachDRB*01 (1.0)	Oct 2013	25	2/25	0.08	138
		Mar/Apr 2014	7[d]	3/7	0.43	137

[a]Infection intensity is the number of Bd Genome Equivalents (GEs), or the number of single-celled Bd organisms, detected in the total pool of DNA extracted from each sample

[b]Head-started frog release site

[c]Seven of nine frogs were head-started individuals

[d]One of seven frogs was a head-started individual

log-transformed replicate qPCR runs was 0.29 (±0.39 SD). Infection prevalence was variable but did not show significant differences among populations (Fisher exact test, $P=0.12$) or sampling months (Fisher exact test, $P=0.29$; Fig. 2a). In contrast, infection intensity was significantly different between populations (Kruskal–Wallis H test, $\chi^2=33.7$, df = 8, $P=0.0004$) and across sampling months (Kruskal–Wallis H test, $\chi^2=18.1$, df = 5, $P=0.0028$; Fig. 2b, c). Across all individuals, the final logistic regression model explaining allele-specific influences on *Bd* infection found that having RachDRB*04 was a significant predictor of being infected with *Bd* (Wald test statistic = 2.084, $P=0.038$), whereas heterozygosity and the presence of any other specific allele were not significant (Online Resources 2 and 3).

After converting PBR alleles into functional supertypes based on discriminant analysis of physiochemical binding properties among peptide-binding amino acids, all *R. chiricahuensis* and *R. yavapaiensis* alleles collapsed into five distinct supertypes (Fig. 1d; Online Resource 4). PBR supertypes corresponded to sets of clades within the PBR genealogy (Fig. 2). Supertypes 2 and 3 were each comprised of two distinct clades and were only recovered from *R. yavapaiensis*, and supertype 5 comprised a single *R. yavapaiensis* allele that split supertype 2 into two clades. In contrast, supertypes 1 and 4 included alleles from both *R. chiricahuensis* and *R. yavapaiensis*. Supertype 1 consisted of one large *R. yavapaiensis* clade and a distinct *R. chiricahuensis* clade containing only two alleles (RachDRB*01 and RachDRB*05). Supertype 4 also consisted of two clades, one with a mix of one *R. chiricahuensis* (RachDRB*03) and six *R. yavapaiensis* alleles, and the other consisting of two *R. chiricahuensis* alleles (RachDRB*02 and RachDRB*04) that form the basal split with all other PBR alleles in the genealogy. We only detected significant signatures of positive selection acting on codon position 46 in our alignment (Fig. 2, green branches). Only three terminal branches showed significant evidence of positive

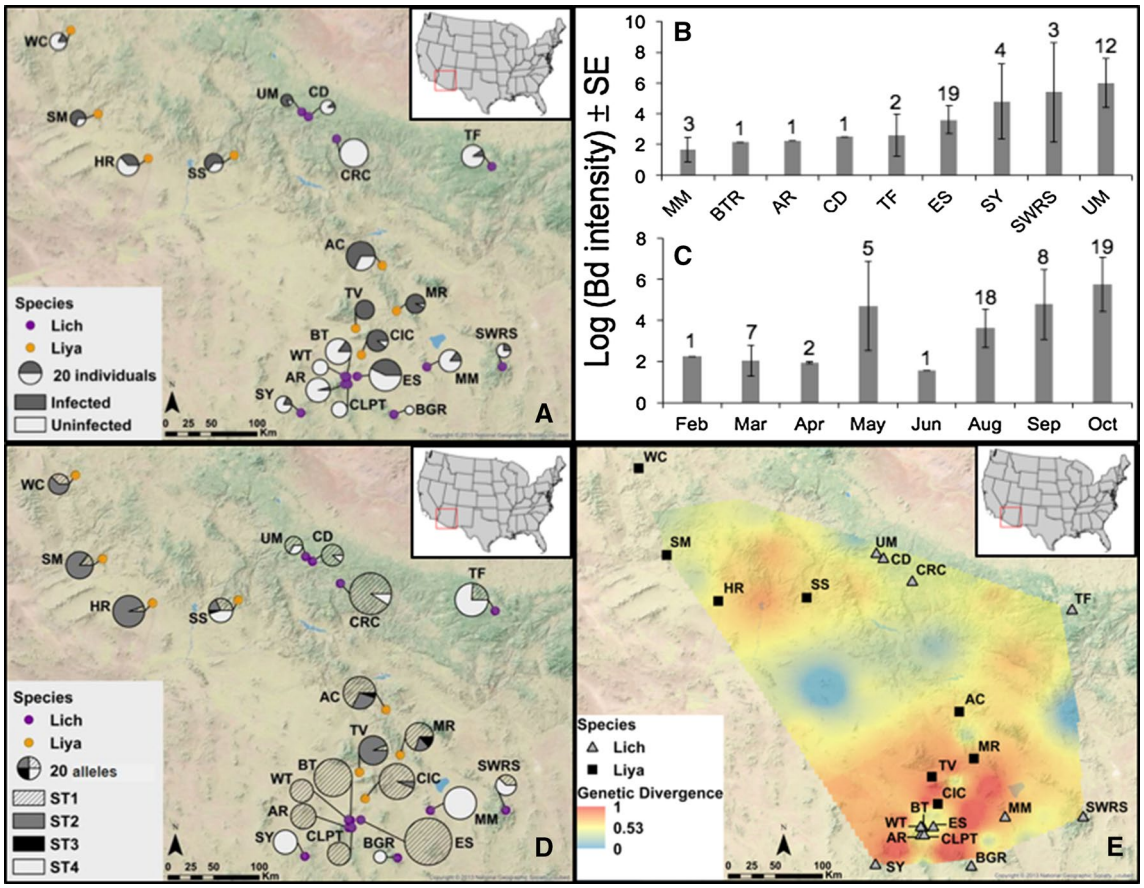

Fig. 2 Spatial patterns *of Bd* infection and immunogenetic connectivity in *R. chiricahuensis* (*purple symbols/triangles*) compared to *R. yavapaiensis* (*yellow symbols/squares*) in Arizona, USA. **a** Proportion of individuals infected with *Bd* across populations; **b** *Bd* infection intensity across *R. chiricahuensis* population localities; **c** *Bd* infection intensity across *R. chiricahuensis* sampling months; **d** MHC supertype frequencies across populations; **e** MHC genetic divergence among *R. chiricahuensis* and *R. yavapaiensis* populations with highly differentiated populations represented by warm colors and minimally differentiated populations represented by cool colors. Circle sizes are proportional to sample size. *AC* Aravaipa Canyon, *AR* Gardner Canyon, *BGR* Beatty's Guest Ranch, *BT* Bowman Tank, *CD* Cabin Draw, *CIC* Cienega Creek, *CLPT* Crazy Lazy P Tank, *CRC* Crouch Creek, *ES* Empire Spring, *HR* Hassayampa river, *MM* middle march, *MR* muleshoe ranch, *SM* Santa Maria River, *SS* Seven Springs, *SWRS* Southwestern Research Station, *SY* Sycamore Canyon, *TF* Three Forks Tank, *TV* Tanque Verde Canyon, *WC* Willow Creek, *WT* West Tank, *UM* Upper Moore. (Color figure online)

selection (Online Resource 5): one of the 84 *R. yavapaiensis* alleles in supertype 2 (a proline to valine), the *R. chiricahuensis* allele RachDRB*01 (a leucine to proline) and the *R. chiricahuensis* allele RachDRB*02 (a proline to valine); all three of these amino acids are non-polar and hydrophobic.

PBR supertype differentiation among *R. chiricahuensis* and *R. yavapaiensis* populations inferred using either F_{ST} or Jost's *D* (Online Resource 6) was significantly higher between species ($D = 0.59$) than within species (*R. yavapaiensis* $D = 0.39$ and *R. chiricahuensis* $D = 0.41$; Kruskal–Wallis H test, $\chi^2 = 168.3$, df = 136, $P = 0.03$). Overall, PBR supertype differentiation showed a weakly significant correlation with Euclidian distance between populations (Pearson's $r = 0.17$; $P = 0.048$). However, neighboring populations in southeastern Arizona were highly differentiated by PBR supertype both within and between species (warm colors), whereas in northern parts of the state populations shared similar PBR alleles and frequencies (cool colors), even between species (Fig. 1e).

Across 2587 bp of mitochondrial 16S and 18S rRNA sequence with one individual sampled per *R. chiricahuensis* and *R. yavapaiensis* population (Online Resource 7), eight haplotypes were recovered from each species, suggesting similar species-wide levels of mitochondrial haplotype diversity (GenBank IDs MF537009–MF537035; Table 2). Although MHC allelic diversity was an order of magnitude higher in *R. yavapaiensis* compared to *R. yavapaiensis*, mitochondrial nucleotide diversity was slightly lower in *R. yavapaiensis* compared to *R. chiricahuensis* (Table 2; Fig. 3). Additionally, among MHC alleles over 57% of sites were variable in *R. yavapaiensis* but only 10%

Table 2 Comparison of genetic diversity recovered from MHC and mitochondrial sequences recovered from *R. chiricahuensis* compared to *R. yavapaiensis*

	R. chiricahuensis	*R. yavapaiensis*
MHC sequences		
Sampled populations	15	8
Number of alleles	5	84
Mean alleles per population	1.4	8.8
Mean pairwise identity	94.3%	93.0%
Identical sites	89.7%	42.5%
Mitochondrial sequences		
Sampled populations	15	12
Number of haplotypes	8	8
Mean pairwise identity	99.5%	99.8%
Identical sites	98.7%	99.5%

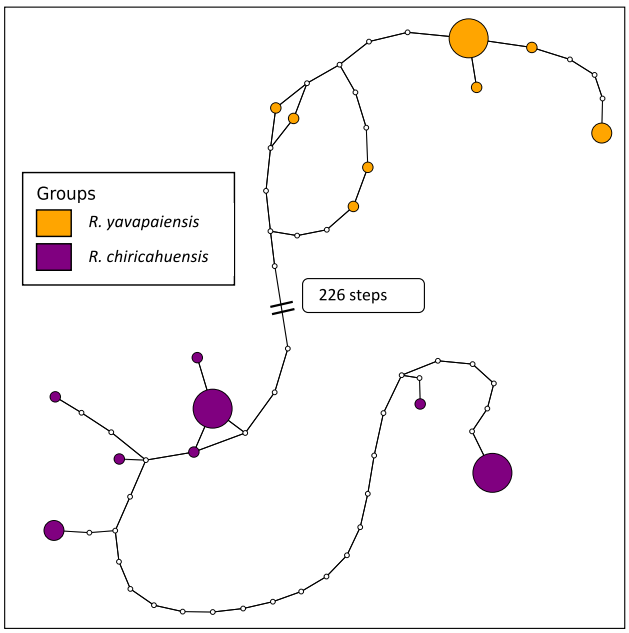

Fig. 3 Haplotype network of 16S and 18S mitochondrial sequences recovered from one individual sampled per population from *R. chiricahuensis* (N = 15; *purple*) and *R. yavapaiensis* (N = 12; *yellow*). *Circle sizes* are proportional to the number of individuals recovered with each haplotype. *Steps* represent single nucleotide changes. (Color figure online)

of sites were variable in *R. chiricahuensis*, whereas among mitochondrial haplotypes 1.3% of sites were variable in *R. chiricahuensis* compared to 0.5% of sites in *R. yavapaiensis* (Table 2).

Pre-release survival while in captivity was over 70% for both of the head started clutches. We toe-clipped individuals that had metamorphosed prior to the release date, including 83 head-started metamorphs reared from a Gardner Canyon (AR) egg mass and 200 head-started metamorphs reared from a Cabin Draw (CD) egg mass. Consistent with natural population sampling from these RUs, AR metamorphs were fixed for PBR allele RachDRB*01(supertype 1), and CD metamorphs harbored two PBR supertypes and genotypes: 55% were RachDRB*03/RachDRB*05 (supertype 4/supertype 1) heterozygotes and 45% were RachDRB*05 (supertype 1) homozygotes, suggesting single paternity and parental genotypes of RachDRB*05/RachDRB*05 and RachDRB*03/RachDRB*05. AR metamorphs were released to a site within the RU they were collected from named Bowman Tank (BT) and CD metamorphs were released to a site within the RU they were collected from named Upper Moore (UM; Fig. 1). At BT, a total of three head-started frogs were recaptured, and only one was recaptured after winter (Table 3). We also toe-clipped 23 resident frogs in October 2013, of which five were recaptured the following spring, and we toe-clipped six resident frogs in spring 2014 that had not previously been sampled (Table 1). Chytridiomycosis die-offs were observed in BT at the end of 2013 and no frogs were detected at this site by June 2014. We did not release CD frogs to Upper Moore until October 2013 due to the later timing of metamorphosis, thus we did not begin surveys until spring 2014. A total of seven head-started frogs were recaptured at Upper Moore in March, April and June of 2014, and four of the seven frogs were recaptured more than once, indicating that at least some frogs were persisting over time (Table 3). This survival cannot be attributed to the absence of *Bd* at this location, as we collected six resident frogs in October 2013 that were dead or manifesting signs of chytridiomycosis and all harbored *Bd* (Table 1). In contrast, none of the recaptured head-started frogs were infected with *Bd* at any recapture time point (Tables 1, 3).

Because all individuals released to BT had the same PBR genotype, we could not assess MHC influences on overwinter survival. However, among UM-released metamorphs, significantly more RachDRB*05 (supertype 1) homozygotes were recaptured (7 out of 89) compared to RachDRB*03/RachDRB*05 (supertype 4/supertype 1) heterozygotes (0 of 111; Fisher exact test, $P = 0.003$; Fig. 4). Because we marked and released all resident frogs encountered during release of head-started frogs and subsequent surveys, we were able to estimate total resident population sizes at each locality. At BT, we marked 23 resident frogs and recaptured six frogs, five of which were marked, giving a population size estimate of 27 resident individuals (95% confidence interval: 20–34). At UM, we marked seven resident frogs and subsequently captured four resident frogs, none of which were marked, giving a population size estimate of 39 resident individuals but a very wide 95% confidence interval (0–85) due to small capture and recapture sizes. Because population size estimates were based on

Table 3 *Rana chiricahuensis* mark-release-recapture surveys from August 2013 through June 2014

Eggmass source	Release location	Release date	No. released	No. recaptures per survey month			
Gardner Canyon (AR)	Bowman Tank (BT)	Aug 2013	53	Oct 2013	Mar 2014	Apr 2014	June 2014
		No. head-started frogs captured (total)		2	0	1	0
		No. head-started frogs recaptured		N/A	0	0	0
		No. resident frogs captured (total)		23	2	4	0
		No. resident frogs recaptured		N/A	1	4	0
Cabin Draw (CD)	Upper Moore (UM)	Oct 2013	197	Oct 2013	Mar 2014	Apr 2014	June 2014
		No. head-started frogs captured (total)		N/A	1	6	4
		No. head-started frogs recaptured		N/A	N/A	1	3
		No. resident frogs captured (total)		7	2	0	2
		No. resident frogs recaptured		N/A	0	0	0

Head-started frogs refer to the frogs reared at the Phoenix Zoo and released to the site; resident frogs refer to frogs naturally occurring at the release sites. Captures refer to the total number of frogs per category that were sampled on each survey date, and recaptures refer to the number of those frogs that had been captured on previous surveys

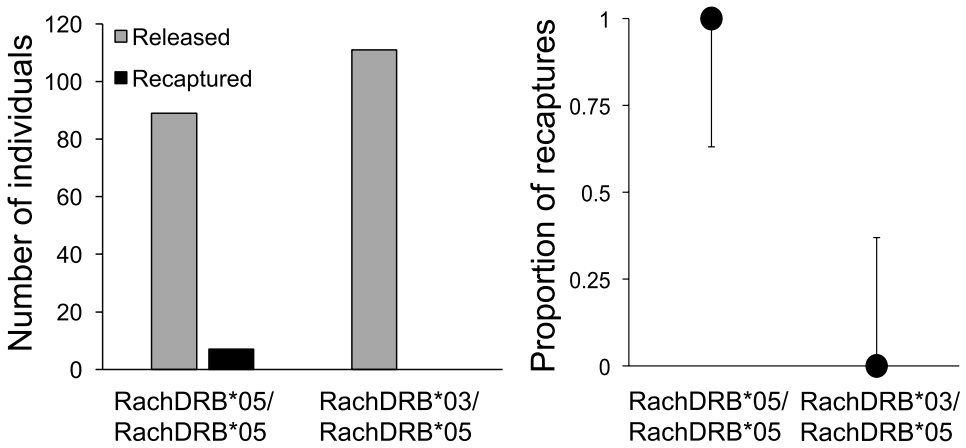

Fig. 4 Proportion of recaptured *R. chiricahuensis* individuals by MHC genotype. **a** Number of head started and released individuals compared to number of recaptured individuals across MHC genotypes. **b** Proportion of each genotype recaptured with 95% exact binomial confidence intervals for prevalence

recapture rates of toe clipped individuals, and toe clipping may reduce the probability of recapturing an individual (Perry et al. 2011a, b), these estimates are conservative.

Discussion

We found strikingly low class IIβ MHC diversity across Arizona *R. chiricahuensis* populations. The presence of five alleles in total and no more than two alleles per population is considerably lower than typical patterns in wild vertebrate populations (Bernatchez and Landry 2003), and is exceptionally low compared to the 84 alleles detected in only eight populations of the congener *R. yavapaiensis* (Savage and Zamudio 2016). Although *R. chiricahuensis* has suffered severe population declines in recent decades (USFWS 2007), the lack of MHC variation we detected cannot be explained by a loss of total genomic polymorphism because the number of mitochondrial haplotypes was identical in *R. chiricahuensis* compared to *R. yavapaiensis*. This contrasting pattern between mitochondrial variation and functional MHC variation suggests that low MHC diversity in *R. chiricahuensis* is not the original cause of high susceptibility to chytridiomycosis (Sredl and Jennings 2005). Instead, low MHC polymorphism within populations is likely a consequence of selection acting on MHC allele frequencies in populations as they declined, causing MHC alleles that conferred a fitness benefit to fix much faster than could be expected due to drift alone (Ejsmond and Radwan 2011; Sutton et al. 2011; Eimes et al. 2011).

Comparing MHC diversity to neutral variation in microsatellite or mitochondrial DNA is a common approach for inferring the type of evolutionary forces responsible for current levels of MHC polymorphism. As expected, variable patterns are recovered across these studies; MHC diversity can be equal to, greater than or less than neutral makers (Sutton et al. 2011), suggesting that selection drives MHC diversity in some cases but neutral forces dominate in others. However, using microsatellites or mitochondrial DNA to infer neutral patterns of diversity is problematic in

that neither marker type is directly comparable to MHC in the mode of sequence evolution or the selective and other forces that may be acting (for a discussion of this topic, see Spurgen and Richardson 2010). Thus, we included range-wide mitochondrial diversity as a snapshot of genomic diversity to determine that low MHC variation in *R. chiricahuensis* could not be explained by a total loss of genetic diversity when compared to *R. yavapaiensis* (Fig. 3). To draw more specific conclusions about the nature of selection acting on MHC alleles, we instead focused on measures of positive selection along particular MHC lineages (Fig. 2) and the experimental survival differences based on MHC genotypes (Fig. 4).

Three of the five *R. chiricahuensis* alleles we recovered grouped more closely with *R. yavapaiensis* alleles than with the other two *R. chiricahuensis* alleles, a phenomenon known as trans-specific polymorphism that is typically attributed to balancing selection maintaining allelic lineages for extended periods of time (Takahata and Nei 1990). Within the *Rana pipiens* complex, *Rana chiricahuensis* and *R. yavapaiensis* are not close relatives (MRCA at least 18 Mya; Yuan et al. 2016), further highlighting that MHC lineages often pre-date multiple speciation events. The only codon we found to be under positive selection in *R. chiricahuensis*, residue 46, is the same positively selected codon in *R. yavapaiensis* that is associated with surviving experimental *Bd* exposure (Savage and Zamudio 2011) and with elevated *Bd* tolerance in natural populations (Savage and Zamudio 2016). *Rana chiricahuensis* residue 46 is one of the 15 residues comprising the mammalian-defined class IIβ exon 2 peptide-binding region (Brown et al. 1993). This codon position is also one of the P9 pocket peptide-binding residues that is under positive selection and associated with experimental *Bd* survival in the frog *Litoria v. alpina* (residue 57 in Bataille et al. 2015). Given these independent lines of evidence linking residue 46 to positive selection and pathogen survival in distinct frog taxa, this codon position is likely a central determinant of MHC class IIβ peptide binding in amphibians. However, PBR alleles RachDRB*03 and RachDRB*05 have the same amino acid at codon 46 (leucine; Online Resources 1 and 5) despite the differences in overwinter survival based on the presence of RachDRB*03, suggesting that it is the combination of PBR amino acids that determines actual pathogen binding potential and not the amino acid present at any single position (Kosch et al. 2016; Didinger et al. 2017).

Terminal branch positive selection acting on residue 46 was detected in 2/5 *R. chiricahuensis* alleles but only 1/84 *R. yavapaiensis* alleles, suggesting that positive selection has had stronger recent impacts on adaptive evolution of MHC alleles in *R. chiricahuensis*. Although a direct measure of differential *Bd* susceptibility between these two species have not been established via common garden experimental exposures, patterns of declines and observations of die-offs indicate *R. chiricahuensis* is the more susceptible species (USFWS 2007; Sredl and Jennings 2005). Furthermore, *R. chiricahuensis* inhabits a higher elevational range than *R. yavapaiensis* (Platz and Mecham 1979), causing more of the year to be spent experiencing cool, chytridiomycosis-promoting temperature regimes, whereas *R. yavapaiensis* spends most of the year under warm, chytridiomycosis-limiting temperatures (Savage et al. 2011). Thus, positive selection for *Bd* tolerance and/or resistance is likely a stronger and more constant evolutionary force in *R. chiricahuensis* compared to *R. yavapaiensis* populations, leading to higher selective pressures and potentially more dramatic loss of MHC polymorphism due to higher average resistance conferred by the remaining alleles. Consistent with this hypothesis, one of the alleles under recent positive selection (RachDRB*01) is the most abundant allele recovered in *R. chiricahuensis* and has gone to fixation in five of the six populations it was recovered from (Table 1).

Most natural Arizona population samples we analyzed were collected in warmer months, when *Bd* infections are significantly lower compared to winter (Savage et al. 2011) and the pathogen is therefore less likely to be detected even if it is present. Nonetheless, we detected *Bd* in nine of 13 sampling localities and across all seasons (Table 1) and found a significant positive association between having RachDRB*04 and being *Bd* infected across all sampled individuals. Seasonal infection dynamics in Arizona have previously been based on *Bd* samples collected from *R. yavapaiensis* (Schlaepfer et al. 2007; Forrest and Schlaepfer 2011; Savage et al. 2011). Infection data recovered in this study therefore suggest that *Bd* infections in *R. chiricahuensis* may be more persistent across seasons, potentially due to the higher elevational range occupied and cooler mean temperatures experienced throughout the year.

Recent positive selection leading to RachDRB*01 (supertype 1; Fig. 2), which is fixed in five *R. chiricahuensis* populations, indicates that directional selection for pathogen survival may have caused this allele to become fixed. However, because RachDRB*01 is already fixed in the AR population where we sourced one of the head-started egg masses, it was impossible to directly test MHC-based survival impacts for this cohort post-release. In contrast, the two distinct genotypes present in the second cohort of head-started frogs sourced from CD enabled us to infer functional consequences of different MHC genotypes. Head-started individuals that were RachDRB*05 homozygotes were significantly more likely to be recaptured in the spring following release compared to RachDRB*03/RachDRB*05 heterozygotes (Fig. 4). These findings are consistent with natural population studies of tungara frogs where directional selection

for *Bd* resistance has also led to fixation of MHC alleles within populations (Kosch et al. 2016). Two caveats to this study are that (1) overall recapture rates were low, indicating low detection probability and/or overall survival, and (2) some individuals may have dispersed from the release site, and heterozygotes may have been more behaviorally prone to dispersal given that MHC molecules are involved in mate choice as well as immunity (Jenions and Petrie 1997). However, despite the small sample size our observed difference in recapture rates was significant, indicating a true difference in overwinter survival based on MHC genotype. We also selected isolated, small ponds as release sites to minimize the probability that any individuals were not recaptured because they left the study site. Thus, the fixation of supertype 1 (RachDRB*01) in nearly half of all sampled populations, as well as the overwinter recapture of only supertype 1 (RachDRB*05) homozygotes from a cohort harboring two alleles from two supertypes (RachDRB*03/RachDRB*05), are two lines of evidence that suggest supertype 1 alleles have undergone recent positive selection selective sweeps because they contribute to chytridiomycosis resistance.

Identifying genetic markers for resistance has the potential to improve ex situ rearing success rates, and may ultimately lead to an ex situ breeding program using marker-assisted selection to generate elevated chytridiomycosis resistance. By exploiting the natural process of genetic disease adaptation known to occur across vertebrate taxa, rather than a more direct interventionist approach, the methods described here have the potential to generate an enormous conservation return on investment. Increasing the frequency of resistance alleles in natural populations via head-starting and/or ex situ breeding may provide natural populations with long-term capacity to tolerate the presence of *Bd*, requiring no further conservation intervention. Although this approach could have unintended negative consequences, such as a reduction in overall local adaptation and in total genetic variation, for extreme cases where disease pressure is causing widespread population extirpations, the benefits of a genetically-informed reintroduction program may far outweigh the costs.

Acknowledgements We thank Ruth Allard, Shaula Hedwall, David Hall, Bradley Poynter, Christina Akins and Michael Sredl for their assistance in planning and implementing this project in Arizona. We also thank Nancy McInerney and Rob Fleischer for supporting data generation at the Smithsonian's Center for Conservation Genomics. This study was funded by an Association of Zoos and Aquariums Conservation Grant Fund award (12-1111) to AES and SW and an Arizona Game and Fish Department award (Central Arizona Project) to AES and SW. KPM was supported by a doctoral student grant (PD/BD/52604/2014) from the Portuguese ''Fundaçao para a Ciencia e a Tecnologia".

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