

MHC genotypes associate with resistance to a frog-killing fungus

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The emerging amphibian disease chytridiomycosis is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). Amphibian populations and species differ in susceptibility to *Bd*, yet we know surprisingly little about the genetic basis of this natural variation. MHC loci encode peptides that initiate acquired immunity in vertebrates, making them likely candidates for determining disease susceptibility. However, MHC genes have never been characterized in the context of chytridiomycosis. Here, we performed experimental *Bd* infections in laboratory-reared frogs collected from five populations that show natural variation in *Bd* susceptibility. We found that alleles of an expressed MHC class IIB locus associate with survival following *Bd* infection. Across populations, MHC heterozygosity was a significant predictor of survival. Within populations, MHC heterozygotes and individuals bearing MHC allele Q had a significantly reduced risk of death, and we detected a significant signal of positive selection along the evolutionary lineage leading to allele Q. Our findings demonstrate that immunogenetic variation affects chytridiomycosis survival under controlled experimental conditions, confirming that host genetic polymorphisms contribute to chytridiomycosis resistance.

emerging infectious disease | experimental infection | major histocompatibility complex | molecular evolution | Ranidae

Amphibians face a biodiversity crisis (1) brought on in large part by the emerging infectious disease chytridiomycosis (2–5). The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) causes chytridiomycosis in amphibian species worldwide, but the extent of morbidity and mortality varies within and among populations (6, 7), species (7), and assemblages (7, 8). Some of this variation is attributable to regional and local ecological factors such as climate (9), elevation (2), host life history traits (10), and different *Bd* strains (11). However, host immunity likely also contributes to disease outcomes. In the model frog *Silurana tropicalis*, experimental *Bd* inoculations significantly change host gene expression profiles (12), and innate (13) and acquired (14) immune responses are activated at host-optimal temperatures. In addition, experimental infections of the toad *Alytes obstetricans* show that *Bd* survival varies among individuals and populations under constant environmental conditions (15), and upon reinfection, previously *Bd*-infected *Leiopelma archeyi* clear the pathogen significantly faster than control animals (16), suggesting a secondary acquired immune response. Identifying heritable determinants of *Bd* resistance has the potential to enhance the success of amphibian conservation efforts (17). However, to date, immunogenetic correlates of survival remain underexplored (18).

Vertebrate MHC genes are good candidates for influencing host *Bd* dynamics because they encode cell-surface glycoproteins that regulate the vertebrate acquired immune response and affect the development and progression of wildlife disease (19). To initiate acquired immunity, MHC proteins bind pathogen molecules on their peptide-binding regions (PBRs) and present them to T cells (20). This central function in pathogen defense creates strong diversifying selection within host populations for numerous MHC proteins that can collectively bind a wide array of pathogens (21). MHC genes are divided into three classes (I, II, and III) that are genomically linked across tetrapods (22). Class I loci are expressed on all nucleated somatic cells and present peptides derived from

intracellular pathogens, whereas class II genes are expressed in epithelial cells in the thymus and antigen-presenting cells in the periphery, and primarily present peptides derived from extracellular pathogens (23, 24). The class III region encodes a group of structurally unrelated proteins that are not involved in antigen presentation (but have immune function) (24). Class II MHC loci are the most likely candidate immunity genes for chytridiomycosis for two reasons: (i) they are the primary presenters of extracellular fungal pathogens (23) and (ii) class II-expressing dendritic and Langerhans lymphocytes are present in amphibian skin (25, 26), the primary location of *Bd* infections. Despite these data, class II genes have only been described in a few amphibians (24, 27–32) and never, to our awareness, in the context of *Bd* susceptibility.

Associations between disease susceptibility and MHC polymorphisms are widespread in natural wildlife populations (19). Most studies focus on variation in the PBR of class I and class IIB loci because PBR amino acid variability determines the repertoire of pathogen peptides to which T cells can respond (20). Across diverse vertebrate taxa, survival after disease exposure has been associated with particular PBR alleles (33), elevated PBR heterozygosity (34), and intermediate levels of PBR diversity (35). These multiple mechanisms of disease resistance indicate that MHC-associated immunity is context-dependent and varies with each combination of host, pathogen, and environment. Chytridiomycosis is caused by a single pathogen affecting numerous amphibian hosts that occupy diverse habitats, thereby providing a unique epidemiological combination for immunogenetic studies of independent hosts evolving under varied environmental regimes.

The lowland leopard frog (*Lithobates yavapaiensis*) is a North American amphibian that began experiencing mass chytridiomycosis die-offs in the early 1990s (6). Currently, populations persist with *Bd* infections that vary in prevalence and intensity across space and time (36, 37). In some populations, a proportion of individuals die from chytridiomycosis during winter months when temperatures are pathogen-optimal (36). In other populations, *Bd* infection prevalence and intensity increase during winter months, but host frogs never develop signs of disease (36). This pattern suggests two nonexclusive hypotheses: (i) genetic resistance to chytridiomycosis occurs in some populations or (ii) environmental variation explains differences in disease dynamics among populations. However, the spatial and seasonal variation in pathogen and disease occurrence across populations precludes distinguishing genetic from environmental determinants of *Bd*-associated mortality in the field.

Here, we determined the role of host genetic variation in *Bd*-associated mortality by experimentally infecting laboratory-

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reared *L. yavapaiensis* collected from five geographically distinct populations that naturally differ in disease dynamics. We exposed individuals to *Bd* and induced chytridiomycosis under constant, pathogen-optimal environmental conditions. We then tested the following four hypotheses: (i) laboratory survival varies across individuals and/or populations in patterns that correspond to observed chytridiomycosis mortality in the field; (ii) pathogen burdens are lower in surviving individuals compared with individuals that die; (iii) genetic variation at MHC loci associates with survival following infection; and (iv) MHC alleles associated with survival show a signature of positive selection. Combined, these hypotheses address the overarching question of whether heritable host immune factors explain differences in *Bd* susceptibility in natural populations.

Results

We characterized natural chytridiomycosis in five *L. yavapaiensis* populations during 2007 through 2010 (Fig. 1A and Fig. S1) (36) to generate a priori expectations for survival patterns in our experimental *Bd* challenges. We collected egg masses in 2008 and reared them through metamorphosis in a light-, temperature-, and humidity-controlled growth chamber. Before the experiment, we tested for, but did not detect, native *Bd* infections among our experimental animals. Metamorphosed frogs were randomly assigned to two replicated treatments: (i) infected with Arizona *Bd* strain PsTr2004 via exposure to 1×10^5 zoospores in liquid media or (ii) kept as uninfected controls exposed to liquid media only. Among the 99 frogs experimentally infected with *Bd*, 14 individuals survived the experiment (Fig. 1B and Table S1); survivors were from Aravaipa Canyon (AC) and the Santa Maria River (SM). These two populations showed 41% and 27% survival in the laboratory, respectively, and are also the two populations with the lowest proportion of mortalities in the field (Fig. 1A). No experimentally infected frogs from Cienega Creek (CIC), Muleshoe Ranch (MR), or Willow Creek (WC) survived, corroborating the hypothesis that laboratory survival varies across populations according to chytridiomycosis mortality in the field. Mean infection intensity, defined as the number of *Bd* genome equivalents recovered per swab, was not significantly different from zero at 1 d post infection (DPI; $P = 0.74$) or 3 DPI ($P = 0.39$), but zoospore counts increased significantly in all populations at 7 DPI ($P = 0.0005$; Fig. 1C and D). At 14 DPI, mean infection intensity was no longer significantly different from zero for individuals from AC and SM that survived ($P = 0.5$; Fig. 1D, open symbols) but remained significantly higher than zero for all individuals that eventually died ($P < 0.0001$; Fig. 1D, closed symbols). Thus, among *Bd*-infected individuals, those that died maintained *Bd* infection intensities higher than zero for the duration of the experiment, whereas surviving individuals cleared *Bd* infections by 14 DPI (Fig. 1C and D).

Population genetic background can bias disease association analyses (38); therefore, we genotyped all 99 *Bd*-infected frogs at 14 unlinked microsatellite loci (39) to characterize population structure for comparison with immunogenetic variation. We implemented a Bayesian assignment test (40) that confirmed the independent evolutionary history and current genetic isolation of each sampled population: frogs were assigned to six genetic demes ($\ln L, -2,984.1$) corresponding to their five natal populations, with further differentiation between clutches from population CIC (Fig. 2A). We next characterized the PBR of an expressed MHC class IIB gene (41) and sequenced PBR genotypes for all *Bd*-infected individuals to test for associations between allelic polymorphisms and responses to *Bd* infection. We recovered 33 unique PBR alleles (GenBank accession nos. JN638850–JN638882; Fig. S2), of which only six occurred in multiple genetic demes. Ten of the 12 sampled clutches showed evidence of multiple paternity based on microsatellite and PBR genotypes (Fig. S3 and Table S1); thus, the majority of clutches included half-sibships, rather than full sibships.

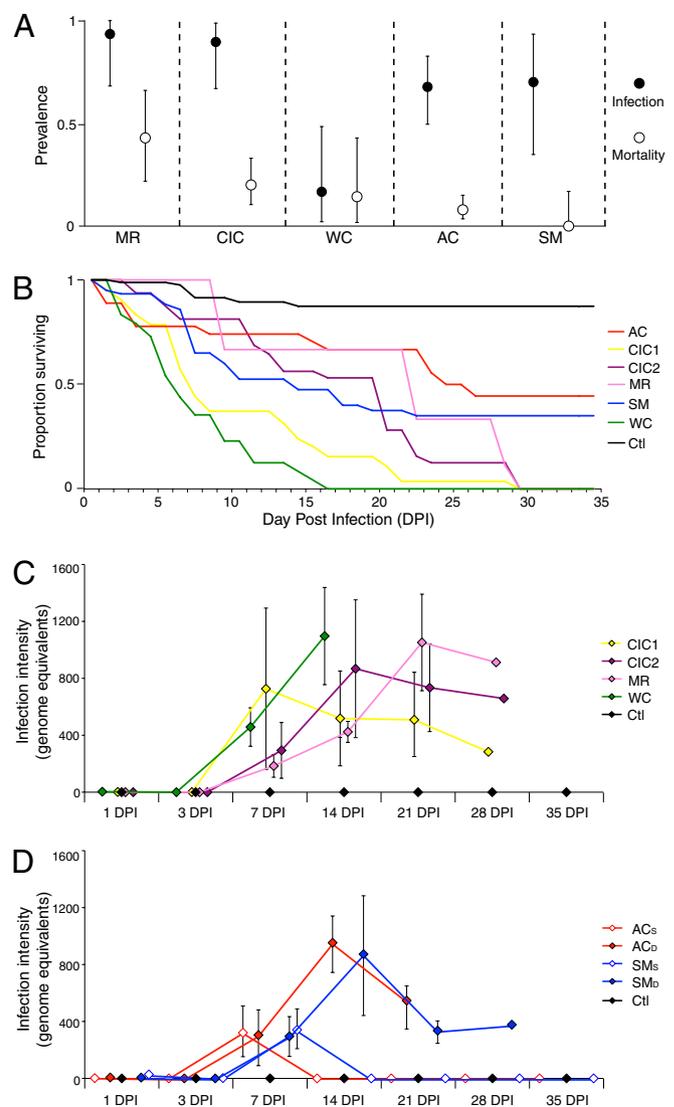


Fig. 1. *Bd* infection and mortality vary among populations of *L. yavapaiensis* in nature and in the laboratory. (A) Field estimates of winter *Bd* infection prevalence (proportion of frogs testing positive for *Bd*; filled symbols) and mortality (proportion of *Bd*-positive frogs that died; open symbols) in five natural populations; 95% Clopper-Pearson binomial confidence intervals are indicated (modified from ref. 37). (B) Survival following *Bd* infection varies among individuals from the same five populations when reared in a growth chamber. Survival was tracked for 35 DPI with 1×10^5 zoospores of *Bd* (colored lines) or sham infection (black line). CIC1 and CIC2 denote genetically distinct clutches from population CIC. Numbers of individuals per population (*Bd*-infected/uninfected) are as follows: AC, 17/11; CIC, 34/22; MR, 5/2; WC, 17/11; SM, 26/15; all, 99/59. (C) Mean *Bd* infection intensity (zoospore equivalents \pm SD) measured at various DPI intervals in populations with no survival (CIC, MR, and WC). (D) Mean *Bd* infection intensity over time for survivors (open symbols; AC_s and SM_s) and nonsurvivors (filled symbols; AC_d and SM_d) from populations with survivors (AC and SM). Control frogs (black symbols) remained uninfected for the duration of the experiment.

To identify parameters with significant effects on survival, we used the Cox proportional-hazards model including treatment, genetic deme, clutch, replicate, number of frogs per replicate, initial mass, change in mass, PBR heterozygosity, maximum infection intensity (MII), and interactions between MII and all other parameters (complete model, $\Delta \ln L = 36.2$, $df = 22$, $P < 0.0001$). As expected, significantly more uninfected individuals survived compared with *Bd*-infected individuals ($P = 0.001$). Among infected individuals, PBR heterozygosity ($P = 0.0002$), MII ($P =$

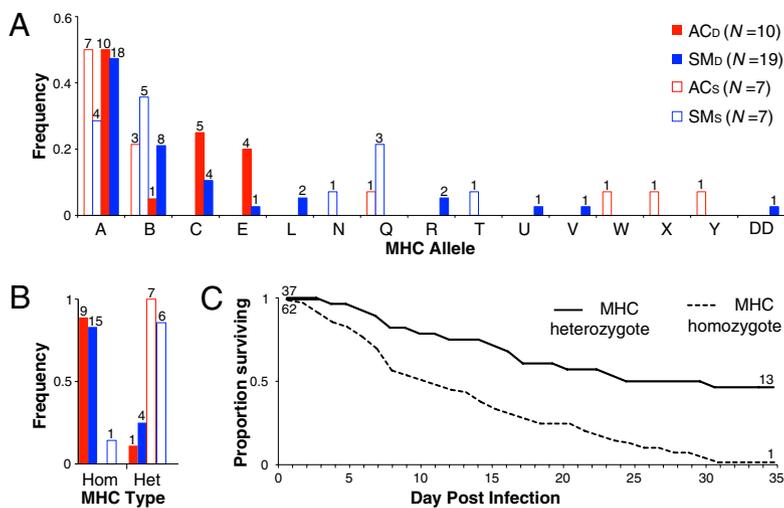


Fig. 3. MHC class IIB PBR allele and heterozygote frequencies for *Bd*-infected survivors and nonsurvivors. PBR allele distribution (A) and PBR heterozygote and homozygote frequency (B) among survivors (open bars) and nonsurvivors (filled bars) in AC (red bars) and SM (blue bars), the two genetic demes with survivors. Allele counts and numbers of heterozygous and homozygous individuals are indicated above frequency bars. (C) Survival curves for PBR heterozygotes (solid line) and homozygotes (dotted line) across *Bd*-infected individuals from all five genetic demes.

cannot exclude the possibility that the class IIB locus examined here shows significant associations with survival as a result of linkage with another locus, because at least two class II genes are expressed in *L. yavapaiensis* (41). However, the signal of positive selection we detected along the PBR lineage leading to the only allele significantly associated with *Bd* survival challenges linkage as the sole explanation for our results. To the contrary, recent positive selection acting in the PBR of a resistance-associated allele found in natural populations provides compelling evidence that this locus encodes a molecule with specific involvement in the immunological response to *Bd*.

Our results underscore the evolutionary advantage of diverse genetic resistance pathways in an ever-changing environment. Lowland leopard frogs show spatial and temporal variation in demography and *Bd* exposure (36), which likely creates population-specific selective regimes for the evolution of chytridiomycosis resistance. Furthermore, host *Bd*-resistance alleles may carry some fitness cost with regard to other *Bd* strains or pathogens (45, 46). Because of the specificity of MHC-peptide binding, individual alleles often provide a fitness advantage against some pathogen epitopes while simultaneously incurring a

fitness cost against others (19, 47). Thus, the strength and targets of selection on the MHC are likely to differ across populations, depending on the genetic background of the host and the full complement of pathogens at each site. In this context, our finding that PBR heterozygosity was a fitness advantage across all populations underscores that the broader peptide binding capabilities inherent to having two MHC alleles can be more advantageous than having one allele with particular efficacy against one pathogen epitope (46). Heterozygote advantage may thus be the general mechanism for enhancing chytridiomycosis resistance in natural populations. However, we also detected a resistance allele in population SM, highlighting that individual alleles can be selectively advantageous for specific genetic backgrounds and environmental regimes. Numerous studies of wildlife populations demonstrate that genetic disease resistance is a host-, strain-, and context-dependent process (19, 45), and our results confirm that lowland leopard frogs have reduced chytridiomycosis susceptibility via multiple evolutionary mechanisms.

The ability of host populations to evolve *Bd* resistance could be limited by the recent timescale of chytridiomycosis emergence. Lowland leopard frogs have suffered from *Bd*-induced declines for only 20 generations (6), and genetic theory predicts that the shorter generation time of pathogens should provide them an advantage in the coevolutionary arms race (47). However, hosts can benefit from high standing genetic diversity, because the rate of pathogen evolution is negatively correlated with host genetic variability (48–50). Empirical studies also demonstrate that relative generation times of hosts and pathogens do not influence local adaptation (51). In fact, when pathogen genetic variability is low, as is the case for *Bd* in the New World (52), short generations increase the strength of selection for host resistance and decrease the capacity of pathogens to adapt to an evolving host (53). Chytridiomycosis in *L. yavapaiensis* thus fits a scenario with strong potential for the evolution of heritable disease resistance in host populations. In our study, SM and AC were the only populations in which individuals varied in *Bd* survival and the only populations with the PBR allele (i.e., Q) showing a signal of recent positive selection. Importantly, these populations also had the lowest proportions of *Bd* mortalities in our field surveys, indicating that our experimental results have biological relevance to *Bd* dynamics in natural populations. These populations are therefore the best candidates for future studies of the evolutionary dynamics of *Bd* resistance in natural populations.

Animal hosts evolve two mechanisms to combat infections: resistance, defined as the ability to limit pathogen burden, and tolerance, the ability to limit the damage caused by a given pathogen burden (54). The distinction between tolerance and resistance is critical for understanding the evolutionary con-

Table 2. Evidence of positive selection among 82 amino acid residues of *L. yavapaiensis* MHC class IIB PBR sequences

Method	LRT for model allowing $\omega > 1$ rate classes*	Percentage of sites in $\omega > 1$ rate classes	Mean ω per $\omega > 1$ rate class
Rate class			
PARRIS	9.16 (0.01)	15%	4.3
Evolutionary fingerprint	26.1 (0.006)	27%	4.4
Codon-specific	Codon 3 dN-dS [†]	Codon 18 dN-dS [†]	Codon 46 dN-dS [†]
SLAC	18.1 (0.09)	—	—
FEL	1.1 (0.03)	0.93 (0.03)	0.74 (0.04)
REL	3.2 (0.99)	3.3 (0.99)	—

dN-dS, normalized difference between nonsynonymous substitutions per nonsynonymous sites (dN) and synonymous substitutions per synonymous sites (dS); FEL, fixed-effects likelihood; LRT, likelihood ratio test; PARRIS, partitioning approach for robust inference of selection; PP, posterior probability; REL, random-effects likelihood; SLAC, single likelihood ancestral counting; ω , dN/dS ratio.

*Values in parentheses are *P* values.

[†]Values in parentheses are *P* values/posterior probability.

sequences of chytridiomycosis disease dynamics because resistance has a negative effect on pathogen fitness and tolerance does not (54). Under our experimental conditions, surviving frogs cleared *Bd* infections, demonstrating disease resistance. However, the same lowland leopard frog populations (AC and SM) show evidence of disease tolerance in the field, where individuals carry high pathogen burdens with no disease signs (36). These differences underscore the need to consider experimental infection studies and disease dynamics in wild populations. Controlled experiments allow us to pinpoint the effects of immunogenetic factors in the absence of environmental variation. However, the critical next step will be studies of natural populations incorporating environmental factors that mediate host resistance and tolerance, pathogen virulence, and population MHC variability.

Given the scale of the amphibian biodiversity crisis (4), understanding host–pathogen chytridiomycosis dynamics is essential for amphibian conservation. Our study shows that genetically distinct lowland leopard frog populations experimentally infected with *Bd* have significant differences in survival that correlate with immunogenetic heterozygosity and polymorphism, and thus that host genetic variability determines *Bd* infection outcomes under controlled environmental conditions. To date, studies of natural amphibian populations have compared chytridiomycosis susceptibility across sympatric species (2, 8, 9), among intraspecific populations (55), and between individuals (56). Our findings emphasize the need for studies that quantify host population genetics and immunogenetics at these same hierarchical levels. For highly susceptible species, captive breeding is one of the few remaining strategies for maintaining natural populations (57). Species reintroductions with assisted selection could be a viable chytridiomycosis mitigation strategy, but will require a better understanding of amphibian immune responses and immunity genes targeted by natural selection (19). Although allele Q may be unique to *L. yavapaiensis*, we also detected significant heterozygote advantage, and selection for MHC heterozygosity is a strategy that could be implemented for any *Bd*-susceptible amphibian species. Here we have identified genetic determinants of chytridiomycosis resistance in natural populations of a declining amphibian, a critical step in developing genetically informed breeding programs for species recovery.

Methods

Experimental *Bd* Exposures. Frogs were infected with *Bd* strain PsTr2004, an Arizona strain isolated from a *Pseudacris triseriata* individual collected from Coconino County, AZ (Universal Mobile Telecommunications System coordinates: 446716E, 3871380N), in 2003. This locality is geographically distinct from our five populations, minimizing the potential that they were locally adapted to this *Bd* strain. Frogs from each replicate group were split into two cages; one cage was seeded with 1×10^5 PsTr2004 *Bd* zoospores in 1 mL of tryptone/hydrolyzed gelatin/lactose (TGhL) broth and the other cage was sham-infected with 1 mL of TGhL broth only. The film of water/TGhL/*Bd* or water/TGhL covering the floor of each cage was left undisturbed for 24 h, and a new film of dechlorinated laboratory water was added to each cage daily. Cages were rotated daily to avoid the potential for microclimatic effects. Frogs were weighed immediately before infection and at death or the end of the experiment, whichever came first.

Infection Intensity Over Time. To quantify *Bd* infections, we used sterile swabs and followed standard amphibian swabbing protocols (58). *Bd* DNA was extracted using PrepMan Ultra, and we used quantitative PCR (59) to determine *Bd* infection intensity. Before experiments began, we tested individuals for preexisting *Bd* infection at all three developmental stages (eggs, larvae, and metamorphs). One day before experimental *Bd* infections, we assayed all metamorphs for *Bd* infection. Frogs were handled with unused latex gloves to prevent *Bd* transmission. Following *Bd* exposures, we quantified infection intensity for each surviving replicate group on 1, 3, 7, 14, 21, 28, and 35 DPI.

Statistical Analysis of Survival. We analyzed survival data by using the Cox proportional-hazards model (60), which assumes an underlying function

describing how hazard changes over time and fits effect parameters using Cox likelihood. Survival was censored, meaning that individuals may die after the end of the study period. To identify potential predictors, we first performed a univariate analysis for each variable and each interaction among variables. We performed log-rank tests of equality across strata for categorical variables (replicate, clutch, source population, number of cage mates, and MHC heterozygosity), and univariate Cox proportional-hazards regression for continuous variables (initial mass, change in mass, and MII). Predictors with a *P* value of 0.1 or less in the univariate analyses were included as potential effect parameters in the final model. Significant *P* values were determined by using sequential Bonferroni correction (61). We tested whether infection intensities were significantly different from zero for survivors and nonsurvivors by using Wilcoxon signed-rank tests. Tests were performed in JMP software, version 9.0 (SAS).

Screening of Microsatellite Loci. We genotyped all *Bd*-infected individuals at 14 previously characterized microsatellite loci (39). DNA was extracted by using a 5% Chelex 100 solution with 0.5 μ g proteinase K per sample. Chelex extractions were incubated at 55 °C for 120 min and 99 °C for 10 min. PCR amplification was performed under the following conditions: 5 min initial denaturation at 94 °C; 35 cycles of 1 min denaturing at 94 °C, 1 min annealing at primer-specific annealing temperatures, 1 min extension at 72 °C, and a final extension of 75 °C for 5 min. Amplified products with different labels or nonoverlapping size ranges were multiplexed and run on a 3730 Genetic Analyzer (Applied Biosystems). Fragment sizes were determined by comparison with a LIZ-500 standard by using GeneMapper, version 3.5 (Applied Biosystems).

Population Structure. Genetic structuring among populations was inferred for all *Bd*-infected individuals. We used the program Structure, version 2.3 (40), to identify the most likely number of genetic clusters (*K*) present and the proportion of membership in those clusters for each *Bd*-infected clutch. This method uses Bayesian assignment techniques to identify clusters of genetically similar individuals from multilocus genotypes without prior knowledge of their population affinities. Twenty independent runs were performed for each value of *K* ranging from 1 to 12, with a burn-in of 1 million Markov chain Monte Carlo iterations and a data collection period of 3 million iterations.

MHC Amplification, Cloning, and Sequencing. We extracted genomic DNA from toe-clips of *Bd*-infected individuals by using Qiagen DNeasy kits. We used a degenerate MHC class IIB forward primer (MHC-F) (31) and an intron-specific reverse primer (B1intron2_R) (41) to amplify 246 bp of exon 2 and 189 bp of the adjacent 3' flanking intron. This primer pair was designed to amplify alleles from a single locus and ensure that only orthologues were compared, because at least two MHC class IIB loci are expressed in *L. yavapaiensis* (41). Subsequent analyses included the exon 2 coding region only. Amplifications were performed for 35 cycles at 95 °C for 50 s, 60 °C for 45 s, and 72 °C for 1 min. We cloned PCR products into the Promega pGEM T vector and transformed recombinant DNA into Invitrogen TOP-10 *Escherichia coli*. Cells were grown on Luria agar plates for 18 to 22 h at 37 °C. We used blue/white screening to choose eight to 32 clones from each transformation and amplified them by using M13 primers with standard reaction conditions. PCR products were purified using an alkaline phosphatase-exonuclease reaction and sequenced on an ABI 3730 sequencer using Big Dye (version 3.1) chemistry. We discarded MHC sequences recovered singly with two or fewer nucleotide polymorphisms to other sequences, attributing these small differences to PCR or cloning error. After discarding these sequences, no more than two alleles were recovered from any individual. We used translated amino acid queries in GenBank to confirm MHC class IIB homology and aligned sequences in Sequencher, version 4.10 (Gene Codes).

Genealogy Reconstruction. We tested the alignment of MHC sequences for recombination by using Genetic Algorithm for Recombination Detection (62), and detected no recombination; therefore, we performed an unrooted maximum-likelihood (ML) analysis. Model parameters were determined using Akaike and Bayesian information criteria in jModeltest, version 0.1.1 (63). The best-fit model (HKY85) was used in a ML analysis in PAUP* (Sinauer) using the heuristic search option, TBR branch swapping, MulTrees option in effect, and a single neighbor-joining tree as a starting topology. Node support was estimated from 1,000 bootstrap replicates.

Tests of Selection. We ran tests of selection using the HyPhy software package (64) with the ML genealogy as our input tree. We used the partitioning

approach for robust inference of selection method to test for positive selection across the alignment, and the evolutionary fingerprinting method to infer positive selection rate classes and selection intensity. We tested for residue-specific selection in PBR lineages using three ML methods: single likelihood ancestral counting (SLAC), fixed-effects likelihood (FEL), and random-effects likelihood (REL) (64).

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