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Spatiotemporal adaptive evolution of an MHC immune gene in a frog-fungus disease system

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Abstract

Genetic diversity of major histocompatibility complex (MHC) genes is linked to reduced pathogen susceptibility in amphibians, but few studies also examine broad spatial and temporal patterns of MHC and neutral genetic diversity. Here, we characterized range-wide MHC diversity in the Northern leopard frog, *Rana pipiens*, a species found throughout North America that is experiencing disease-related declines. We used previously sequenced neutral markers (mitochondrial DNA and microsatellites), sequenced an expressed MHC class II β gene fragment, and measured infection prevalence and intensity of the global fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) across 14 populations. Four populations were sampled across two decades, enabling temporal comparisons of selection and demography. We recovered 37 unique MHC alleles, including 17 that were shared across populations. Phylogenetic and population genetic patterns between MHC and neutral markers were incongruent, and five MHC codon positions associated with peptide binding were under positive selection. MHC heterozygosity, but not neutral marker heterozygosity, was a significant factor explaining spatial patterns of *Bd* prevalence, whereas only environmental variables predicted *Bd* intensity. MHC allelic richness (AR) decreased significantly over time but microsatellite-based AR did not, highlighting a loss of functional immunogenetic diversity that may be associated with *Bd* selective pressures. MHC supertype 4 was significantly associated with an elevated risk of *Bd* infection, whereas one supertype 2 allele was associated with a nearly significant reduced risk of *Bd*. Taken together, these results provide evidence that positive selection contributes to MHC class II β evolution in *R. pipiens* and suggest that functional MHC differences across populations may contribute to disease adaptation.

Introduction

Infectious diseases in wildlife populations are increasing globally and are linked to species declines, threatening biodiversity and conservation (Daszak et al. 2000; Blanchong et al. 2016). Because standing genetic diversity provides the immediate foundation for populations to adapt,

allelic polymorphism can mitigate negative impacts brought on by emerging infectious diseases (McKnight et al. 2017). Specifically, immune gene diversity within the major histocompatibility complex (MHC) is a key component of adaptive variation that can reduce disease impacts in natural populations (e.g., Wegner et al. 2003; Oliver et al. 2009). MHC loci occur in all jawed vertebrates and encode a diverse suite of proteins with a central role in immune defense, particularly acquired immune function (Kaufman 2018). The MHC includes two major classes (I and II) that encode cell-surface glycoproteins that recognize and bind to pathogens at their peptide-binding regions (PBR) and present them to T cells, initiating an acquired immune response (e.g., Bernatchez and Landry 2003). Classical class I MHC proteins are expressed on the surface of most cells and bind peptides from intracellular pathogens, such as viruses, whereas class II MHC genes are expressed on antigen presenting cells and primarily bind and present extracellular pathogen peptides, such as bacteria and fungi (Braciale et al. 1987). Many studies have found that higher diversity

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at MHC loci is linked to reduced pathogen susceptibility (e.g., Radwan et al. 2010; Blanchong et al. 2016) and underlies disease adaptation in numerous vertebrates (e.g., Bernatchez and Landry 2003; Acevedo-Whitehouse and Cunningham 2006; Blanchong et al. 2016).

MHC genes typically exhibit high allelic polymorphism and sequence diversity and harbor multiple functional gene copies within classes I and II (Kaufman 2018). In general, more MHC alleles and gene copies are associated with increased immunity because hosts with more diverse peptide-binding capabilities can eliminate a broader suite of pathogens (Bernatchez and Landry 2003; Spurgin and Richardson 2010; Radwan et al. 2020). However, excessive MHC diversity can act to dampen immune responses (Migalska et al. 2019). Three nonexclusive and empirically supported hypotheses can explain the extreme polymorphism of MHC genes: (1) heterozygote advantage, (2) negative frequency-dependent selection (sometimes termed rare-allele advantage), and (3) fluctuating selection in time and space (Spurgin and Richardson 2010). MHC diversity is beneficial under the heterozygote advantage hypothesis because heterozygous individuals can recognize more antigens and protect against a broader array of pathogens. Indeed, MHC heterozygotes often (but not always) have higher resistance to infections (e.g., Penn et al. 2002; but, see Ilmonen et al. 2007). Under the negative frequency-dependent selection hypothesis, novel or rare alleles have a selective advantage because pathogen populations rapidly evolve resistance to common MHC alleles (Takahata and Nei 1990; Bernatchez and Landry 2003; Phillips et al. 2018). Lastly, fluctuating selection can also explain the maintenance of high MHC allelic diversity if there are differences in pathogen strains and abundances based on environment, location, and time (Hedrick 2002; Osborne et al. 2017). Under this scenario, different MHC alleles confer a selective advantage and increase in frequency in different populations, preserving high MHC diversity across populations (e.g., Teacher et al. 2009). Because all three modes can act to shape overall MHC diversity, identifying the specific selective processes driving adaptation to a particular pathogen is challenging and requires extensive data on genetic structure, MHC diversity, ecology, and disease in natural populations.

Understanding disease resistance is particularly important in amphibians because emerging infectious diseases have led to drastic population declines (Echaubard et al. 2015; Lips 2016; McKnight et al. 2017). Globally, the most pervasive disease threat is from the infectious disease chytridiomycosis, caused by the fungi *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (Berger et al. 1998; Martel et al. 2013), which impacts over 500 species (Scheele et al. 2019). Although the number of functional MHC class II loci and the extent of copy number

variation remain uncharacterized in most frogs, class II diversity is significantly associated with *Bd* susceptibility in several taxonomically distinct species (Bataille et al. 2015; Savage and Zamudio 2016; Fu and Waldman 2017). Several frogs in the genus *Rana* show MHC class II disease associations in natural populations. For example, *Rana yavapaiensis* experimentally exposed to *Bd* show significant associations between *Bd* survival, MHC class II heterozygosity, and specific MHC class II alleles (Savage and Zamudio 2011) at a single, constitutively expressed class II locus across frog species (Kiemnec-Tyburczy et al. 2010). Additionally, MHC class II genotypes at this same locus are significantly associated with overwinter survival in *Rana chiricahuensis* released into *Bd*-endemic ponds (Savage et al. 2018). This relationship between MHC diversity and disease susceptibility is not limited to *Bd*, as *Rana sylvatica* larvae show significant differences in *Ranavirus* infection intensities across MHC genotypes (Savage et al. 2019). In addition to MHC diversity, higher genetic variation at neutral markers has also been associated with lower disease risk in *R. yavapaiensis* (Savage et al. 2015) and other natural anuran populations (Allentoft and O'Brien 2010; Kosch et al. 2016; Horner et al. 2017). However, few studies have simultaneously examined MHC and neutral genetic patterns across space and time.

Leopard frogs are ideal study organisms to examine landscape-scale questions integrating population genetics, immunity, and disease because they are widespread, well-studied, and extensively affected by emerging infectious diseases (Woodhams et al. 2008; Echaubard et al. 2015). The northern leopard frog (*Rana pipiens*) is broadly distributed, ranging throughout southern Canadian provinces and most of the continental United States. A range-wide mitochondrial DNA (mtDNA) study investigating phylogeographic patterns identified that *R. pipiens* was split into populations containing discrete eastern and western haplotypes demarcated by the Mississippi River (Hoffman and Blouin 2004a). Moreover, western populations exhibited reduced mtDNA diversity relative to eastern populations, a pattern confirmed with microsatellites (Phillipsen et al. 2011). Although relatively mobile, *R. pipiens* exhibited distinct patterns of isolation-by-distance (Hoffman and Blouin 2004a; Hoffman et al. 2004; Waraniak et al. 2019) and temporal stability of genetic structure in eastern populations, suggesting that at least those populations were not undergoing contemporary extinction and recolonization events (Hoffman et al. 2004). This baseline of information on genetic structure and evolutionary history of *R. pipiens* provides a robust framework for determining relationships between recent demographic processes, disease, and adaptive genetic diversity.

Contemporary populations of *R. pipiens* have experienced significant declines in the western part of the species

range (Leonard et al. 1999; Hoffman and Blouin 2004b), especially in the southwestern USA (Clarkson and Rorabaug 1989; Rogers and Peacock 2012). *Bd* has negatively impacted *R. pipiens* (Parris et al. 2006; Woodhams et al. 2008; Voordouw et al. 2010) as well as other frogs in the southwestern USA (e.g., Savage and Zamudio 2011, 2016; Savage et al. 2018). Arizona shows a striking pattern where most *R. pipiens* populations have severely declined or been extirpated, but one metapopulation is thriving, possibly owing to introgression of eastern *R. pipiens* genes into this area (O'Donnell et al. 2017). O'Donnell et al. (2017) suggest that *Bd* resistance explains the success of this metapopulation, and that genetic resistance may occur due to eastern *R. pipiens* introgression of MHC variants or skin peptides that are not typically found in western populations. However, investigations of immune gene variation have not been performed across eastern or western *R. pipiens* populations.

Here, we characterized evolutionary history and population genetic diversity of an expressed MHC class II β gene in natural populations of *R. pipiens* across the species range and across two decades to evaluate neutral, selective, and disease-associated processes shaping immune gene diversity. First, we conducted phylogenetic analysis and functional supertyping of MHC alleles to examine spatial and functional distribution of MHC clades. Second, we examined site- and branch-specific patterns of molecular evolution to identify individual codons under positive selection. Third, we compared MHC-derived population genetic

parameters to values previously characterized at neutral genetic loci to assess the role of demography compared to selection in driving MHC diversification across populations. Fourth, for a subset of populations with temporal sampling spanning 1971–2018, we compared population genetic parameters from MHC and neutral loci to test whether significant genetic change had occurred over time. Finally, we measured *Bd* infection prevalence and intensity from all sampled individuals and tested the relative importance of neutral genetic diversity, functional immunogenetic diversity, and environmental factors in explaining spatial patterns of *Bd*. Taken together, these analyses help elucidate the demographic, environmental, spatial, and pathogen-associated factors that have shaped immunogenetic diversity in a widespread, declining amphibian species.

Methods

Field sampling and DNA extraction

We sampled tissues from adult *R. pipiens* hand captured from 14 natural populations throughout North America (Fig. 1). Most populations were sampled in 1999–2001 for Hoffman and Blouin (2004a). Additionally, we used previously unpublished samples from Ohio, USA (Table 1, PLK) and samples collected during two different time periods at localities in Arizona, USA (1999 and new samples collected for this study in 2017–2018), Ontario, CAN (1979

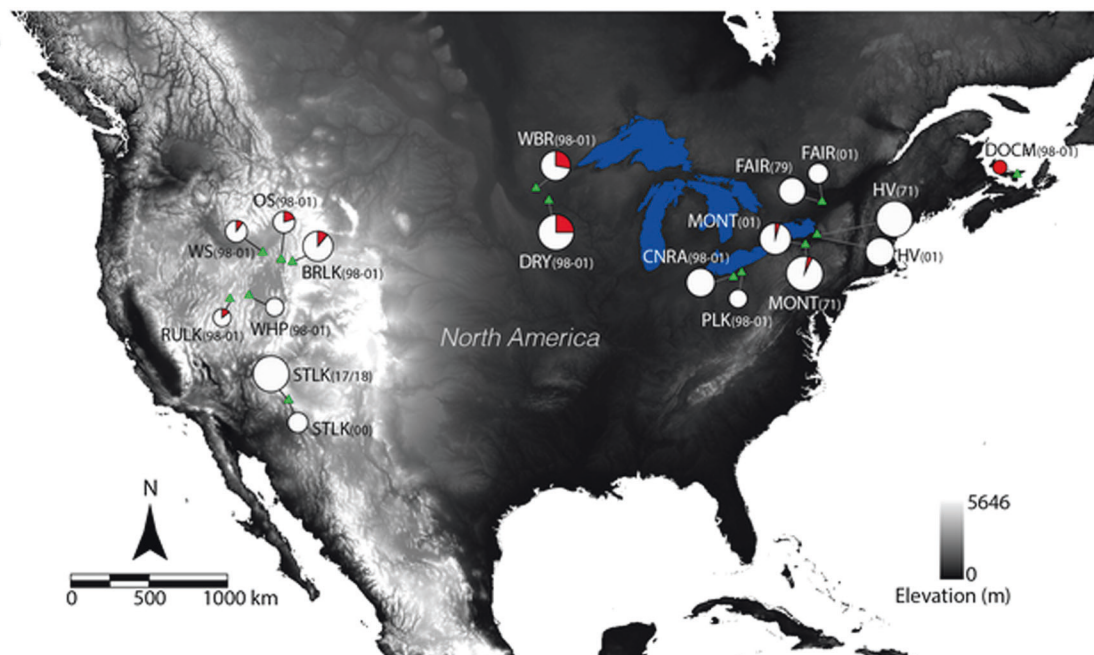


Fig. 1 A map of *Bd* prevalence across sampled *Rana pipiens* populations indicated by green triangles. Circle size is proportional to sample size, with white fill showing the proportion uninfected and

red showing the proportion infected with *Bd*. Population abbreviations follow Table 1 and the year of sampling is in parentheses for each population (color figure online).

Table 1 Sample location, year sampled, and counts of each population for each marker type (MHC, mtDNA, and microsatellites).

Population (year sampled)	State/province	Lat	Long	N		
				MHC	mtDNA	Microsatellites
RULK (1998–2001)	Nevada	40.2482	−115.4711	7	6	–
WHP (1998–2001)	Nevada	40.4299	−114.1840	8	6	–
BRLK (1998–2001)	Idaho	42.1385	−111.2623	18	12	22
OS (1998–2001)	Idaho	42.2568	−112.0131	10	–	23
WS (1998–2001)	Idaho	42.6120	−113.2473	10	–	19
DRY (1998–2001)	Minnesota	45.1264	−94.0306	24	–	37
WBR (1998–2001)	Minnesota	45.7097	−94.8931	18	12	32
PLK (1998–2001)	Ohio	41.5923	−81.0556	8	–	29
CNRA (1998–2001)	Ohio	41.3616	−81.6019	17	12	25
DOCM (1998–2001)	Prince Edward Island	46.3609	−62.5287	6	12	–
STLK (2000)	Arizona	34.8459	−111.5300	9	10	–
STLK (2017/2018)	Arizona	34.8459	−111.5300	26	–	–
FAIR (1979)	Ontario	45.0687	−75.6530	14	–	39
FAIR (2001)	Ontario	45.0687	−75.6530	9	12	38
HV (1971)	New York	43.4679	−76.0100	26	–	54
HV (2001)	New York	43.4679	−76.0100	16	–	22
MONT (1971)	New York	42.9893	−76.7715	24	–	42
MONT (2001)	New York	42.9893	−76.7715	22	12	46
Total				272	94	428

Population names with year sampled in parentheses, along with state/province where each population is located. The latitude and longitude are listed for each population. The number (*N*) of individuals of which we have data for each different marker type (MHC, mtDNA, microsatellites) is listed with dashes indicating no data for a marker in that population.

and 2001), and New York, USA (1971 and 2001). At each site, we preserved a toe clip in ethanol from each captured individual. We extracted genomic DNA from all toe clips using either phenol-chloroform extractions or Qiagen DNeasy extraction kits. For sample sites characterized in previously published studies, we standardized population abbreviations for clarity (Table S1).

MHC genotyping

Using newly collected samples and a subset of tissues from Hoffman and Blouin (2004a), we amplified a 272 bp fragment of a single MHC class II β locus (hereafter, MHC). We used a fusion primer approach designed by Mulder et al. (2017) to amplify and sequence exon 2 of a single MHC locus by targeting the flanking introns. First, we used the MHC primers ForN and RevA (Table S2; Mulder et al. 2017) in a 20 μ L reaction using 5–50 ng of template DNA, 0.9X of OneTaq PCR buffer, 1 mM dNTPs, 0.8 μ g/ μ L BSA, 2% DMSO, 0.16 μ M of each primer, and 0.25 U OneTaq DNA polymerase (New England Biolabs). PCR conditions included initial denaturation of 95 $^{\circ}$ C for 3 min, 35 cycles of 95 $^{\circ}$ C for 30 s, annealing at 52 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C extension for 1 min, followed by 72 $^{\circ}$ C for 5 min. We then

performed a second PCR using Nextera-style Illumina fusion primers combining Illumina adapters, an 8 bp barcode, a primer pad and linker, and the MHC primers used in the first PCR (Table S2). We used unique barcode combinations across forward and reverse fusion primers so that each individual had a unique molecular tag. Fusion primer PCRs consisted of 25 μ L reactions using 4 μ L of the MHC PCR product, 1X OneTaq PCR buffer, 0.2 mM dNTPs, 0.4 μ M of each primer, and 0.625 U OneTaq with an initial denaturation of 95 $^{\circ}$ C for 3 min, 15 cycles of 95 $^{\circ}$ C for 30 s, annealing at 52 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 1 min, followed by a final extension period at 72 $^{\circ}$ C for 5 min. All samples were visualized on 2% agarose gels and grouped by eye into three pools of strong, medium, and weak PCR band intensity. Pools were cleaned with 1.0X SpeedBeads (Thermo Fisher Scientific, Waltham, MA) and quantified using KAPA qPCR kits (Roche Sequencing Systems, Pleasanton, CA). Quantified pools were equimolarly combined into sequencing libraries. Sequencing was accomplished across two Illumina MiSeq 2 \times 250 bp runs, with 77 samples in the first run and 229 in the second. We conducted two independent PCR reactions for two individuals and sequenced them on two different runs to account for possible PCR and sequencing biases.

After demultiplexing, paired-end reads were joined using PEAR v 0.9.8 (Zhang et al. 2014), then clustered, filtered, and genotyped with AmpliSAS (Sebastian et al. 2016). We set the maximum number of alleles per amplicon at two because these MHC primers amplify a single locus (Kiemnec-Tyburczy et al. 2010; Mulder et al. 2017). We set the minimum amplicon depth at 200, and our clustering parameters followed recommendations for Illumina data (Sebastian et al. 2016): 1% substitution errors, 0.001% indel errors, and 25% minimum dominant frequency. For filtering, we set the minimum amplicon frequency to 10%. To further ensure that we only analyzed real MHC alleles, we only retained alleles that were recovered in at least two different individuals.

MHC evolutionary relationships and functional supertypes

We used MEGA7 (Kumar et al. 2016) to create an alignment, translate it, and identify any stop codons. We ran PartitionFinder v2.1.1 (Lanfear et al. 2017) to determine the best model of evolution for the nucleotide alignment. Next, we reconstructed a genealogy in MrBayes v3.2.6 (Ronquist et al. 2012) using *Nanorana parkeri* (Accession Number: KR535970.1) and *Xenopus laevis* (Accession Number: D13688.1) MHC as outgroups. We ran two MrBayes runs of 5×10^6 generations each with the first 10,000 trees discarded as burn-in. We analyzed the output in Tracer v1.7 (Rambaut et al. 2018) to confirm stationarity and sufficient posterior sampling. We also built a maximum likelihood genealogy with IQ-TREE v1.6.9 (Nguyen et al. 2014). Additionally, we created a 95% parsimony MHC haplotype network using the TCS algorithm (Clement et al. 2000) implemented in PopArt v1.7 (Leigh and Bryant 2015). Finally, we created a genus-wide MHC genealogy based on an alignment of all *Rana* (after Yuan et al. 2016) class II alleles in GenBank (identified with the search term “MHC class II Ranidae”) using the same alignment, evolutionary model, and phylogenetic reconstruction methodologies described above, with the same *X. laevis* MHC allele as the outgroup.

We converted MHC alleles into functional supertypes based on quantifiable physiochemical properties of the amino acids comprising the mammalian-defined PBR of each allele (Brown et al. 1993). This analysis does not include phylogenetic relationships among alleles, instead grouping supertypes only based on functional similarity of PBR amino acids. We converted an alignment of these amino acid positions across all alleles into a matrix with five parameters for every amino acid, using the following physiochemical descriptor variables: z1 (hydrophobicity), z2 (steric bulk), z3 (polarity), z4, and z5 (electronic effects; Jombart et al. 2010). We then analyzed this matrix using discriminant analysis of principle components (DAPC)

implemented in the R package adegenet 1.4-0 (Jombart and Ahmed 2011). We defined functional MHC supertypes as all alleles falling into distinct clusters after DAPC. The optimal number of clusters was determined using a change in the Bayesian Information Criterion (ΔBIC) value ≤ 2 .

MHC molecular evolution

We used our *R. pipiens* MHC alignment and the Bayesian output tree file to run HyPhy (Kosakovsky Pond et al. 2005) implemented on the Datamonkey server (Kosakovsky Pond and Frost 2005a). We ran model selection to determine the best model of evolution, then tested for intragenic recombination using SBP (single breakpoint) and Genetic Algorithms for Recombination Detection (GARD; Pond et al. 2006). Next, we tested for codon-based positive selection by estimating synonymous (dS) and non-synonymous (dN) substitution rate parameters using the following six methods: SLAC, FEL, iFEL, REL (Kosakovsky Pond and Frost 2005b), MEME (Murrell et al. 2012), and FUBAR (Murrell et al. 2013). We set the significance threshold at a P value ≤ 0.05 or a posterior probability ≥ 0.95 , depending on the method. We considered codons to be significantly under positive selection if the majority of methods (at least four) found evidence of positive selection. We identified overlap between the codon positions we found to be under positive selection in our data set relative to the mammalian-defined PBR residues (Brown et al. 1993) and to other studies of codon-specific selection in frog MHC (Savage and Zamudio 2011; Bataille et al. 2015).

Spatial comparisons of MHC and neutral loci

The data we analyzed for mtDNA (Hoffman and Blouin 2004a) and microsatellites were derived from a previous study (Hoffman et al. 2006) and from two populations in Ohio, USA (PLK and CNRA) that were originally collected, extracted, and genotyped in 2001, but were previously unpublished. We generated MHC genotypes from a subset of the same samples (as well as the new 2017–2018 STLK sampling), thus we had microsatellite data, mtDNA data, or both for nearly every individual that we MHC genotyped. In this study, we report on seven microsatellite loci (Rpi100, Rpi101, Rpi103, Rpi106, Rpi108, RP193, and RP415) generated under the conditions in Hoffman et al. (2006). We tested for Hardy–Weinberg equilibrium (HWE) using exact tests in Genepop version 3.3 (Raymond and Rousset 1995) for the complete microsatellite data set (genotypes from previously published and unpublished populations). We calculated genetic diversity and differentiation estimates for MHC alleles, MHC supertypes, mtDNA haplotypes, and microsatellites. Although we did not have mtDNA and microsatellite data for all populations,

we had at least one neutral marker type for all populations with MHC sequence data. To estimate expected heterozygosity (H_E , a.k.a. gene diversity; Nei 1987), allelic richness (AR), and pairwise F_{ST} from MHC genotypes, MHC supertypes, and microsatellites, we used the program FSTAT v2.9.3 (Goudet 1995). To estimate observed heterozygosity (H_O), H_E , pairwise F_{ST} , and Tajima's D from mtDNA haplotypes and MHC alleles, we used the program Arlequin v3.5 (Excoffier and Lischer 2010). To test whether H_O and H_E were significantly different from each other for the MHC locus and for microsatellite loci, we used Welch's two sample t -tests (R Core Team 2013). To evaluate significant differences between MHC-derived and microsatellite-derived pairwise F_{ST} estimates, we conducted 5000 bootstrap replicates of all 78 pairwise population F_{ST} estimates for the MHC and microsatellite data sets and evaluated overlap between the means of the bootstrapped distributions (Code File S1). To test for correlations between MHC-derived, microsatellite-derived and mtDNA-derived F_{ST} , we conducted independent single Mantel tests using PASSaGE v. 2.0 (Rosenberg and Anderson 2011) for each pairwise combination of F_{ST} values, with Holm–Bonferroni sequential corrections to adjust for multiple comparison probabilities. To evaluate whether H_O and H_E derived from neutral loci and from MHC were significantly correlated, we measured Pearson's correlation coefficients using the *rcorr* function in the R package Hmisc (R Core Team 2013). We also used effective population size (N_E) previously estimated for a subset of our microsatellite data set (Phillipsen et al. 2011; $N_{E(ABC, 10000)}$) and estimated Pearson's correlation coefficients between N_E , the number of MHC alleles, and the number of private MHC alleles.

To compare spatial connectivity among populations for MHC and neutral loci, we generated two connectivity indices: Euclidean distance (minimum straight-line distance between each population pair, using a least cost function to avoid lines crossing the Great Lakes) and a spatial metric of topographic resistance, estimated by employing our topographic complexity raster as a resistance grid using CIRCUITSCAPE v. 4.0 (McRae et al. 2013). We employed a cell connection scheme linking each node to four neighbors. Spatial connectivity analyses weighed all possible paths between population pairs and produced pairwise resistance matrices and summary connectivity rasters. To avoid the likely bias of multicollinearity in partial mantel tests (Cushman et al. 2013; Guillot and Rousset 2013), we used independent single Mantel tests to correlate Euclidean distance and topographic resistance with pairwise F_{ST} values (calculated separately for MHC, microsatellites, and mtDNA) using PASSaGE v. 2.0 (Rosenberg and Anderson 2011). We used Holm–Bonferroni sequential corrections to adjust for multiple comparison probabilities.

Temporal comparisons of MHC and neutral loci

To evaluate temporal shifts in MHC and neutral marker diversity metrics among the four populations sampled two decades apart, we tested whether MHC AR, H_E , and H_O changed significantly over time using paired t -tests. Similarly, for the three populations with temporal microsatellite data, we tested whether microsatellite AR, H_E , and H_O changed significantly over time using paired t -tests. We did not conduct this test for mtDNA diversity metrics because we lacked mtDNA data for at least one timepoint from all four populations with temporal MHC data.

Genetic and environmental predictors of *Bd* infection

We tested for *Bd* following qPCR protocols from Boyle et al. (2004) using the CFX96 Real-Time system (Bio-Rad). We performed our amplifications in a 25 μ L reaction using 0.64X Supermix (Bio-Rad), 0.8 μ M of each primer (ITS1-3 Chytr: 5'-CCTTGATATAAATACAGTGTGCCATATGTC-3' and 5.8 S Chytr: 5'-TCGGTCTCTAGGCAACAGTTT-3'), 0.2 μ M of probe (Chytr MGB2: 5'-CGAGTCCAAC-3'), 3 μ L molecular-grade H₂O, and 10–100 ng of template DNA. Standard curves were generated using serially diluted gBlocks (Integrated DNA Technologies) and run in duplicate ranging from 2×10^6 to 2×10^{-1} gene copies/reaction (Horner et al. 2017). The qPCR conditions included an initial denaturation of 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Negative and positive controls were run in duplicate with each run. Positives were run at least twice, including a third run if the first two runs were inconsistent by at least an order of magnitude. All values were log transformed to normalize the data and then averaged to obtain infection intensity.

We performed generalized linear model (GLM) selection procedures to identify which genetic diversity metrics and environmental variables best predicted *Bd* prevalence (binomial distribution/logit link) and log-transformed *Bd* infection intensity (normal distribution/identity link) throughout sampled *R. pipiens* populations. We only included population sampling from 1998 to 2001 for GLMs to ensure we were not confounding spatial *Bd* dynamics with temporal variation in pathogen exposure.

To include environmental data, we obtained the following information for each sampling location at 1 km resolution: normalized difference vegetation index (NDVI), 19 bioclimatic variables of temperature and precipitation from Worldclim/Bioclim extrapolated from a global network of climatic stations collecting data from 1970 to 2000 throughout the world (Fick and Hijmans 2017), and elevation from the Consultative Group on International Agricultural Research Consortium for Spatial Information (Jarvis et al.

2009). We generated a topographic complexity raster for North America by employing a moving-window approach, calculating the elevation variance for each pixel based on the eight neighboring 1 km pixels with high values denoting more complex topography. We used principal components analysis to consolidate cross-correlated temperature and rainfall variables and used the first two PC scores of temperature variables and the first two PC scores of consolidated rainfall variables in our model selection procedure. Additionally, because of high cross-correlation among MHC genetic diversity metrics (Fig. S2) and among neutral genetic diversity metrics (Fig. S3), we only included one MHC variable and one neutral variable in our models. Specifically, we used MHC H_E (estimated from FSTAT) and microsatellite H_E (except for the populations lacking microsatellite data, in which case we used mtDNA-based H_E). Thus, our two model selection procedures (one for *Bd* prevalence and one for intensity) included the following 11 explanatory variables: MHC H_E , neutral H_E , latitude, longitude, elevation, topographic complexity, NDVI, temperature PC1, temperature PC2, rainfall PC1, and rainfall PC2. Competing models were ranked based on corrected Akaike Information Criterion (AICc) and we reported one of the most parsimonious models (based on Δ AICc) for each of our response variables. We tested residuals in our most parsimonious models including log-transformed *Bd* prevalence and intensity data using Shapiro–Wilk W test and confirmed normal distribution of residuals (*Bd* prevalence: $W = 0.936$, $P = 0.248$; *Bd* intensity: $W = 0.947$, $P = 0.390$).

To test whether MHC H_E , MHC supertypes, or MHC alleles were associated with the likelihood of acquiring a *Bd* infection, we calculated the relative risk of infection for each supertype and allele that occurred at sufficient frequency ($N \geq 10$), and for MHC heterozygotes compared to homozygotes. Specifically, we calculated relative risk of *Bd* infection as the ratio of the probability of infected individuals with a certain genotype versus individuals without that genotype, using the R package *epitools* (Aragon 2008) and two-sided Fisher's exact tests to infer significance.

Results

MHC genotyping

We obtained high-quality MHC sequence data from 272 individuals including 50 sampled in 1971, 14 sampled in 1979, 182 sampled in 1998–2001, and 26 sampled in 2017–2018 (Fig. 1 and Table 1). The two individuals sequenced in both runs had identical MHC genotypes across runs. MHC amplified consistently across all individuals, and we found no evidence of more than two alleles per

individual, suggesting minimal PCR artefacts arising from null alleles or amplifying multiple gene copies. We recovered a total of 37 unique MHC alleles based on complete exon 2 sequences and partial flanking introns (GenBank Accession Numbers: MN984182–984218). We recovered an average of 4.5 (± 2.6) alleles per population, ranging from 1 to 10 alleles.

MHC evolutionary relationships and functional supertypes

JC + I + G (Jukes and Cantor 1969) was the best model of evolution. Bayesian and maximum likelihood analyses produced similar topologies, therefore we only present the Bayesian genealogy (Fig. 2). MHC alleles did not group into clades based on population or geographic region (Fig. 2). All MHC alleles clustered into four functional supertypes (Fig. S1), and supertype 1 included only *Rapi**32 and *Rapi**33, whereas other supertypes included at least nine alleles (Fig. 2). Supertypes 1 and 3 formed monophyletic groups, but supertypes 2 and 4 were distributed throughout the genealogy (Fig. 2). MHC alleles were polyphyletic relative to mtDNA haplotype groups: four alleles (*Rapi**03, *Rapi**10, *Rapi**32, and *Rapi**33) were shared across eastern and western mtDNA haplotypes (Fig. 2). The haplotype network further illustrates that MHC alleles do not cluster by geographic region (Fig. 3). For example, allele *Rapi**03 occurred in frogs from Arizona, Ontario, and New York, and alleles *Rapi**32 and *Rapi**33 were recovered from Ohio, Idaho, Nevada, Minnesota, and Arizona. Overall, 17 alleles were shared among multiple populations and 20 alleles occurred in only one population. All populations harbored at least two MHC alleles, except for DOCM (Prince Edward Island), which was fixed for a single, private MHC allele (*Rapi**17), and WS (Idaho), which was fixed for a single allele (*Rapi**04) present in three additional populations. Both of these alleles belong to supertype 4. Phylogenetic analysis including all other *Rana sp.* MHC alleles in GenBank (Fig. S4) demonstrates that *R. pipiens* MHC alleles show extensive trans-species polymorphism (TSP), clustering with alleles from nine other species that occur in North America, Central America, Europe, and Asia.

MHC molecular evolution

Model F81 was the best fit model of evolution for our MHC codons, and neither SBP nor GARD analyses found evidence of intragenic recombination. Significant positive selection occurred in five MHC codons based on at least four of the six methods we implemented (Table S3). All five of these codons align to mammalian-defined PBR residues (Brown et al. 1993; Fig. S5).

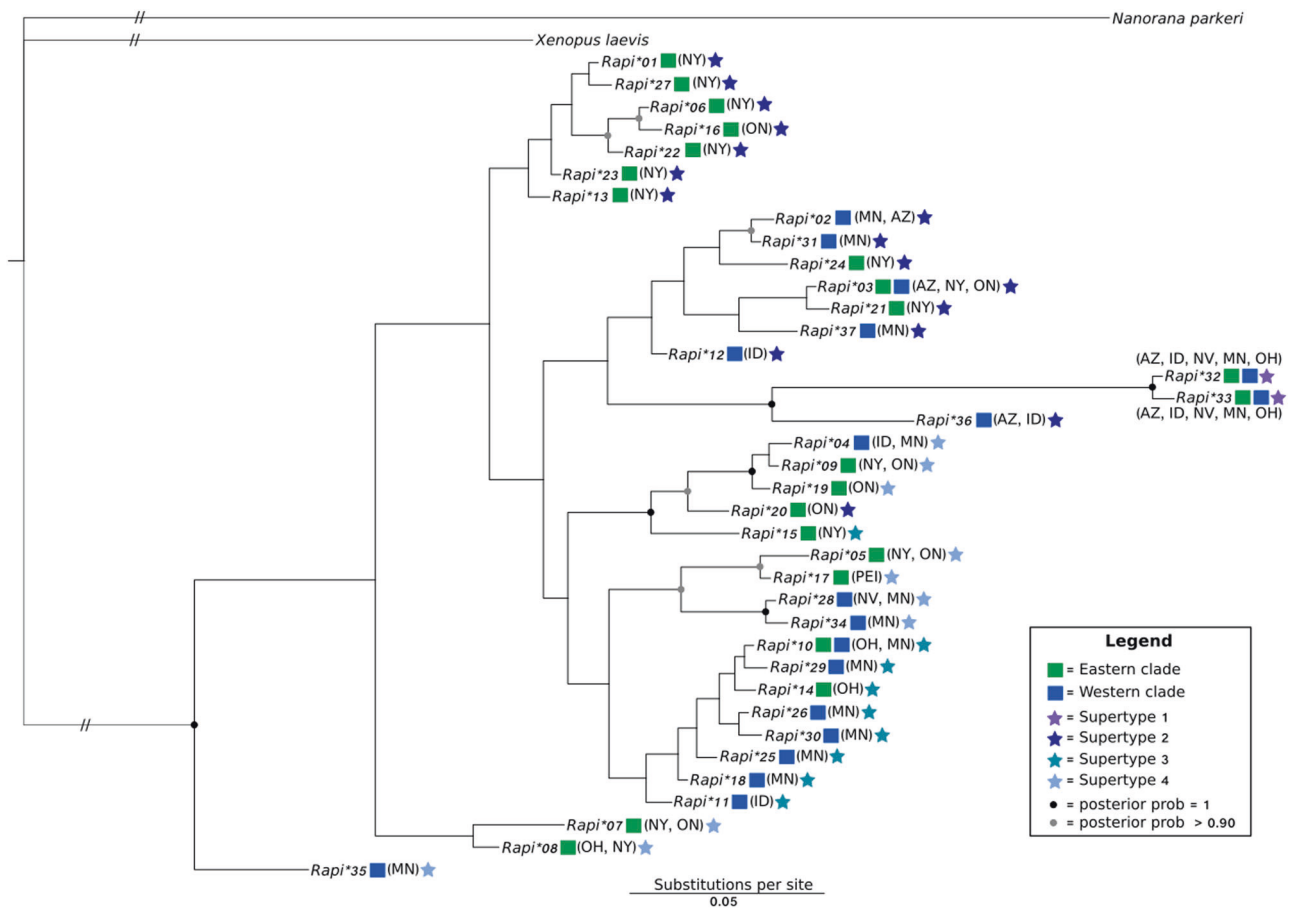


Fig. 2 Bayesian gene genealogy of recovered *Rana pipiens* MHC alleles. Node posterior probabilities are reported when $P > 0.7$. The colored squares indicate previously defined mtDNA clades (see text)

and supertypes are depicted with colored stars. Abbreviations of states/provinces where each allele was found is listed in parentheses (color figure online).

Spatial comparisons of MHC and neutral loci

The mtDNA data came from 94 individuals, including 84 that we MHC genotyped (all collected from 1998 to 2001), and the microsatellite data came from 428 individuals (96 sampled in 1971, 39 sampled in 1979, and 293 sampled in 1998–2001; Table 1), including 216 that we MHC genotyped. For microsatellites, exact tests revealed that only 3/91 comparisons deviated from HWE expectations after a sequential Bonferroni correction (Rice 1989). Specifically, Rpi108 deviated significantly from HWE in the 1971 populations from HPV, and both time points for MONT. Because of the rarity of loci out of HWE, we included all microsatellite loci from all populations in downstream analyses. Across sampled populations, H_O and H_E were not significantly different for microsatellite loci ($t = -1.83$, $N = 13$, $P = 0.093$), but H_O was significantly lower than H_E for the MHC locus ($t = -4.59$, $N = 18$, $P = 0.00029$; Table 2). Tajima's D estimated from MHC was significantly positive in six populations and not significant in all other populations, whereas Tajima's D estimated from mtDNA

was significantly negative in three populations and all other values were nonsignificant (Table 2). Pairwise F_{ST} values estimated from mtDNA versus microsatellite data were not significantly correlated, despite showing a positive trend ($r = 0.296$, $N = 10$, $P = 0.048$, adjusted Holm–Bonferroni threshold for significance = 0.012; Table S5). Likewise, pairwise F_{ST} values estimated from mtDNA versus MHC were not significantly correlated ($r = 0.374$, $N = 28$, $P = 0.077$; Fig. 4A and Table S5) nor were pairwise F_{ST} values estimated from microsatellite versus MHC ($r = 0.331$, $N = 78$, $P = 0.075$; Fig. 4B and Table S5). H_E measured from MHC versus mtDNA data was also not significantly correlated ($r = 0.470$, $N = 9$, $P = 0.200$), but H_E estimated from MHC versus microsatellites showed a significant, positive correlation ($r = 0.710$, $N = 13$, $P = 0.007$). In contrast, H_O estimated from MHC was not significantly correlated with H_O estimated from microsatellites ($r = 0.252$, $N = 13$, $P = 0.407$). Overall, MHC-derived F_{ST} estimates were significantly larger than microsatellite-derived F_{ST} estimates (no overlap across 5000 bootstrapped mean pairwise F_{ST} estimates; Fig. 4C). However,

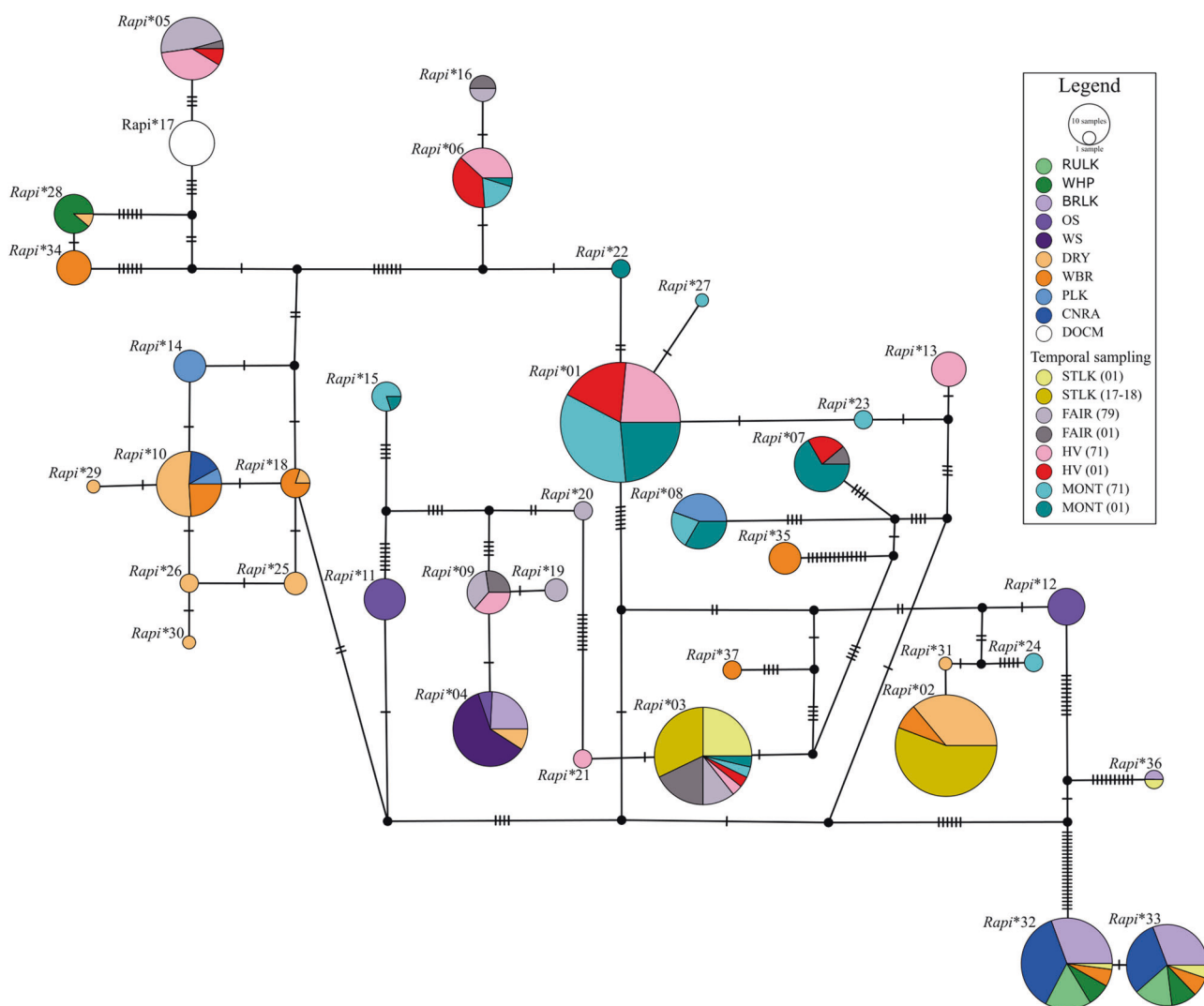


Fig. 3 Ninety-five percent Parsimony haplotype network of *Rana pipiens* MHC class II β alleles created in PopArt. Circles (and names outside circles) represent distinct haplotypes and pie sizes depict

number of samples per haplotype. Colors indicate location of each allele. Black dots represent inferred haplotypes and dashes represent mutations between haplotypes (color figure online).

specific population comparisons showed that MHC-derived F_{ST} was equal to or less than microsatellite-derived F_{ST} for some comparisons (Fig. 4D). For populations with microsatellite-based N_E estimates (Tables 2 and S4), there were no significant correlations between N_E and the number of MHC alleles ($r = 0.194$, $N = 10$, $P = 0.591$) or private alleles ($r = -0.102$, $N = 10$, $P = 0.779$). However, the two populations with the smallest N_E estimates also had the fewest MHC alleles (Table S4).

Mantel tests showed significant positive correlations between Euclidean distance and topographic resistance compared to microsatellite-derived F_{ST} , and for Euclidian distance compared to mtDNA F_{ST} (Table S5). In contrast, neither Euclidean distance nor topographic resistance was significantly correlated with MHC-derived F_{ST} (Table S5). Furthermore, the slopes of associations were considerably

steeper for microsatellite data compared to both mtDNA and MHC data (Fig. S6).

Temporal comparisons of MHC and neutral loci

Comparing the populations sampled approximately two decades apart, we did not detect significant temporal change in MHC H_E ($t = -0.236$, $N = 4$, $P = 0.828$) or MHC H_O ($t = -1.072$, $N = 4$, $P = 0.456$), but we did detect a significant decrease in MHC AR over time ($t = -3.854$, $N = 4$, $P = 0.031$), with the average number of alleles decreasing from 6.25 to 4.75 (Fig. 5). Notably, this included the three northeastern populations sampled in the 1970s versus 1998–2001, as well as the Arizona population sampled in 1999 versus 2017–2018, suggesting a temporal effect of allele loss that is not specific to a particular location or time

Table 2 Effective population size (N_E), expected heterozygosity (H_E), observed heterozygosity (H_O), allelic richness (AR), and Tajima's D for each marker at every site.

Population (year sampled)	N_E	H_E				H_O		Allelic richness		Tajima's D	
		MHC	mtDNA	Microsats	Supertype	MHC	Microsats	MHC	Microsats	MHC	mtDNA
RULK (1998–2001)	–	0.500	0.733	–	0.000	0.857	–	2.000	–	1.43	0.31
WHP (1998–2001)	–	0.679	0.000	–	0.571	0.500	–	2.999	–	3.19	0.00
BRLK (1998–2001)	96	0.683	0.167	0.718	0.408	0.722	0.679	3.307	7.180	0.98	–1.14
OS (1998–2001)	49	0.644	–	0.703	0.644	0.000	0.717	2.853	6.906	1.01	–
WS (1998–2001)	35	0.000	–	0.593	0.000	0.000	0.667	1.000	5.461	0.00	–
DRY (1998–2001)	1137	0.727	–	0.903	0.591	0.375	0.896	4.854	12.593	0.99	–
WBR (1998–2001)	478	0.900	0.621	0.888	0.766	0.277	0.888	6.522	11.955	1.65	0.77
PLK (1998–2001)	–	0.679	–	0.869	0.571	0.000	0.836	2.950	11.869	2.82	–
CNRA (1998–2001)	–	0.599	0.318	0.833	0.221	0.588	0.814	2.841	8.116	–0.35	–1.22
DOCM (1998–2001)	–	0.000	0.000	–	0.000	0.000	–	1.000	–	0.00	0.00
STLK (2000)	–	0.410	0.200	–	0.306	0.222	–	3.235	–	–0.09	–2.03
STLK (2017/2018)	–	0.471	–	–	0.000	0.000	–	1.997	–	3.19	–
FAIR (1979)	3412	0.813	–	0.894	0.495	0.071	0.861	5.084	13.132	1.91	–
FAIR (2001)	4436	0.701	0.318	0.893	0.486	0.222	0.887	4.446	13.338	1.05	–1.75
HV (1971)	4719	0.798	–	0.884	0.388	0.231	0.838	5.169	12.923	1.78	–
HV (2001)	132	0.708	–	0.861	0.325	0.000	0.849	4.087	11.642	0.17	–
MONT (1971)	103	0.631	–	0.916	0.304	0.167	0.805	4.666	13.894	–0.15	–
MONT (2001)	4861	0.723	0.758	0.913	0.530	0.227	0.843	4.358	14.269	0.94	–1.60

Dashes indicate missing data for a specific molecular marker. Tajima's D values shown in bold italics are significant (P value < 0.05).

frame. We did not detect a significant change in microsatellite H_E ($t = -1.281$, $N = 3$, $P = 0.328$), microsatellite H_O ($t = -1.191$, $N = 3$, $P = 0.307$), or microsatellite AR ($t = -0.443$, $N = 3$, $P = 0.701$) over time. Only allele *Rapi**03 occurred in all four temporally sampled populations (Fig. 5). This allele had a very low frequency (below 0.1) in the New York populations, but was present at moderate to high frequencies (0.2–0.8) in Ontario and Arizona.

Genetic and environmental predictors of *Bd* infection

Eight of 14 sites were infected with *Bd* during 1998–2001 sampling (Fig. 1 and Table S6). Average *Bd* prevalence ranged from 0.05 (MONT) to 1.0 (DOCM) with a mean of 0.24. Average infection intensity ranged from 40.7 (OS) to 35,706 (DOCM) genome equivalents with a mean of 12,498 ($\pm 12,575$ SD). Average intensity across all positive individuals was 16,497 ($\pm 20,349$ SD). Model selection indicated that higher MHC H_E predicted lower *Bd* prevalence ($\beta = -4.574$, $\chi^2 = 15.466$, $P < 0.0001$; Tables S7 and S8), along with temperature and precipitation variables. *Bd* infection intensity, however, was best explained by environmental variables only (i.e., rainfall, temperature, and latitude; Tables S7 and S8). Topographic complexity was a positive predictor of *Bd* prevalence ($\beta = 0.001$, $\chi^2 = 16.725$, $P < 0.0001$) and latitude was a positive predictor of

both *Bd* prevalence ($\beta = 1.128$, $\chi^2 = 41.786$, $P < 0.0001$) and intensity ($\beta = 1.011$, $t = 5.890$, $P < 0.0001$). Among MHC alleles that occurred in at least ten individuals, none showed significantly elevated or reduced risk of *Bd* infection. However, allele *Rapi**03 showed a threefold reduced risk of *Bd* infection (relative risk = 0.32) at $P = 0.088$, and none of the 31 individuals with *Rapi**03 were infected with *Bd*. Among supertypes occurring in at least ten individuals, supertype 4 homozygotes showed a significantly elevated risk of *Bd* infection relative to all other supertype genotypes (relative risk = 2.3, $P = 0.029$).

Discussion

Multiple studies in anurans have compared differentiation at MHC genes to neutral loci to establish whether selection contributes to contemporary immunogenetic patterns. The majority of these studies find discordant patterns between MHC and neutral loci (e.g., Savage and Zamudio 2016; Savage et al. 2018; Belasen et al. 2019), confirming that neutral demographic processes alone are insufficient to explain MHC genetic structure. However, these studies typically examine a limited number of natural populations within a species, and none examine MHC and neutral genetic patterns over time. Here, we demonstrate discordant patterns of MHC relative to neutral genetic diversity across

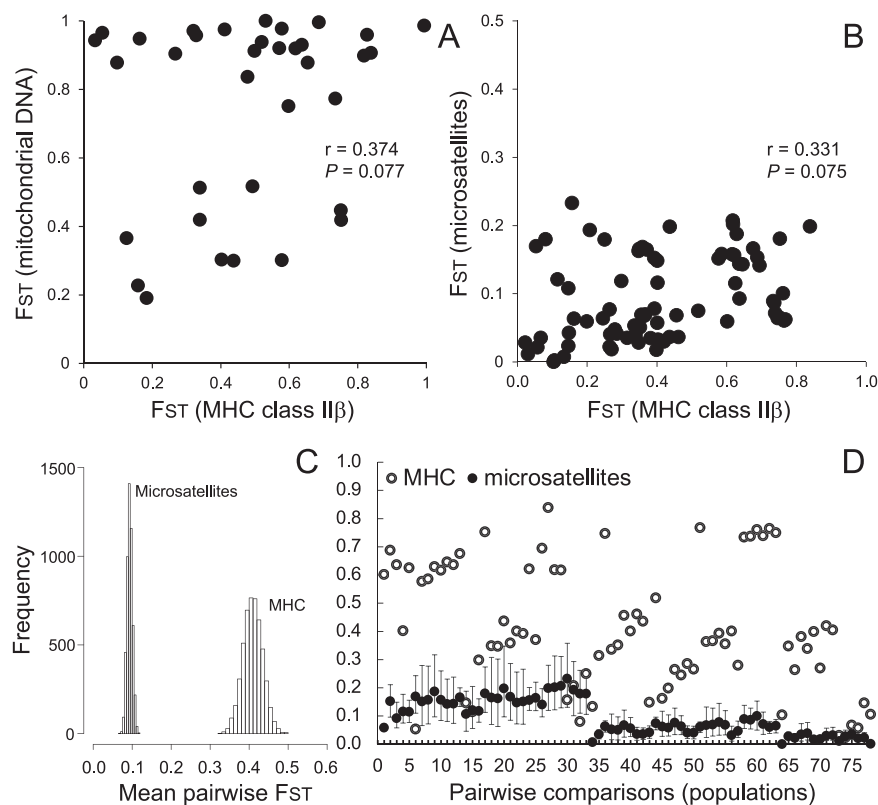


Fig. 4 Comparison of pairwise population genetic differentiation measured across loci for *Rana pipiens* populations sampled in 1998–2001. Pairwise F_{ST} estimates derived from MHC class II β allele frequencies are plotted against pairwise F_{ST} estimate derived from mitochondrial haplotype frequencies (panel (A)) and from microsatellite allele frequencies (panel (B)). Mantel test r values and P values are shown for each comparison. Panel (C) shows the

distribution of 5000 bootstrapped replicates of mean pairwise F_{ST} estimates derived from microsatellites (left) and the MHC locus (right). Panel (D) depicts comparisons of F_{ST} estimates of neutral loci (microsatellites) to MHC class IIB for each pairwise comparison of 11 of our populations. Error bars indicate 95% confidence intervals and were estimated using bootstrapping.

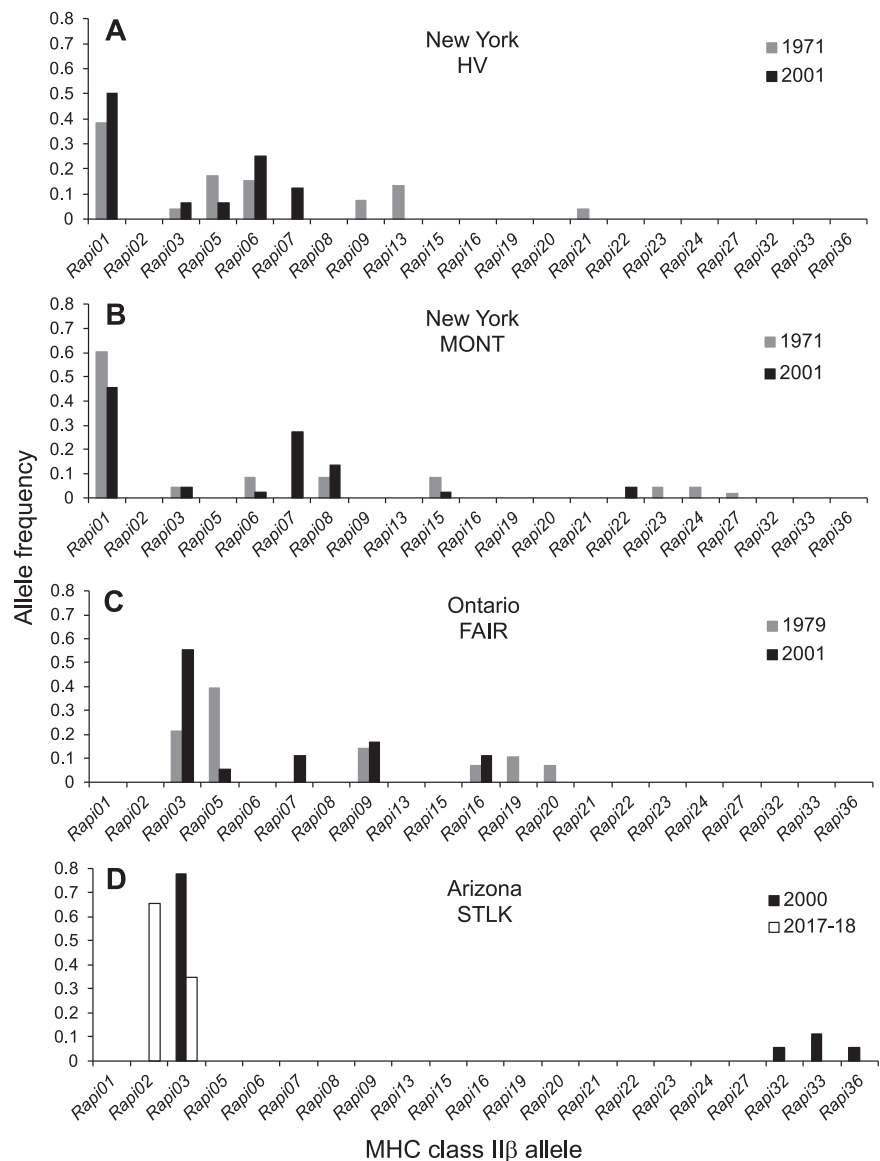
the species range, as well as significant temporal changes in MHC but not neutral AR. These changes coincide with North American chytridiomycosis emergence (Skerratt et al. 2007), suggesting that *Bd* may have contributed to MHC evolution over the past 50 years. The distribution of our 37 *R. pipiens* MHC alleles across every well-supported major clade of the *Rana*-wide MHC genealogy (Fig. S4) demonstrates extensive TSP (Klein et al. 1998), which has been documented for MHC loci in diverse taxa including other amphibians (Bos and Dewoody 2005), fish (Ottova et al. 2005), and mammals (Cutrera and Lacey 2007). Because many *R. pipiens* alleles group with MHC alleles from species with South American and Asian distributions, these TSP are unlikely to have arisen from introgression, and instead represent ancestral polymorphisms maintained by balancing selection preventing the loss of rare alleles.

Spatial comparisons of MHC and neutral loci

Spatial analyses of MHC differentiation typically show contrasts between neutral- and MHC-derived genetic patterns to

infer whether adaptive processes shape MHC evolution (reviewed in Bernatchez and Landry 2003; Radwan et al. 2010). A common trend found in a wide variety of taxa, including mammals (e.g., Cammen et al. 2011), birds (e.g., Strand et al. 2012), fish (Aguilar and Garza 2006), and amphibians (Li et al. 2016), is an overall pattern of neutral markers (e.g., microsatellites) exhibiting lower F_{ST} estimates than MHC-derived F_{ST} . These studies provide evidence that natural selection is favoring different MHC alleles across populations, increasing differentiation relative to levels produced from demographic processes alone. However, a few studies find the opposite pattern of lower MHC-derived F_{ST} estimates, and attribute low MHC differentiation as evidence of balancing selection or homogenizing directional selection (e.g., Evans et al. 2010; Fraser et al. 2010). In *R. pipiens*, mean MHC-derived F_{ST} estimates were consistently larger than microsatellite-derived F_{ST} estimates across 5000 bootstrapped replicates (Fig. 4C), but some population comparisons showed the opposite pattern (Fig. 4D), suggesting balancing selection as well as some homogenizing directional selection. We found a positive correlation between MHC- and

Fig. 5 Temporal shifts in MHC class II β allele frequencies for two time periods across four *Rana pipiens* populations. (A) HV, New York, USA in 1971 vs. 2001; (B) MONT, New York, USA in 1971 vs. 2001; (C) FAIR, Ontario, CAN, 1979 vs. 2001; and (D) STLK, Arizona, USA in 2001 vs. 2017-18. Vertical axes denote the frequency of each allele (along X-axis) in temporally sampled populations (population abbreviations follow Table 1).



microsatellite-derived H_E estimates, suggesting that neutral processes partially contribute to MHC genetic structure. However, MHC F_{ST} estimates were not significantly correlated with Euclidean distance or topographic resistance, and the slopes of association for Mantel tests were steeper for neutral compared to MHC F_{ST} estimates, highlighting that selection on MHC alleles counteracts demographic processes.

Temporal comparisons of MHC and neutral loci

We had a unique opportunity to measure MHC evolution over time using repeat sampling from four populations, each across a 20-year span. Microsatellite-based AR, H_E , and H_O in the three northeastern populations remained stable over time, thus the significant loss of MHC-based AR over time

provides direct observation of directional selection. To our knowledge, only one experimental study in sticklebacks has also demonstrated directional selection by showing that MHC alleles conferring pathogen resistance increased in frequency in a single generation (Eizaguirre et al. 2012). However, other temporal MHC studies recover different patterns. For example, Oliver and Pierny (2012) showed that water voles experiencing a severe population bottleneck recovered MHC (but not microsatellite) diversity within 2 years. Additional long-term studies comparing patterns of MHC genetic diversity over time in populations with and without selective pressure from pathogens may help to resolve the role of directional selection in shaping MHC diversity over ecological rather than evolutionary timescales.

Genetic and environmental predictors of *Bd* infection

Previous studies documented *Bd* infections in *R. pipiens* (Woodhams et al. 2008; Voordouw et al. 2010), but genetic predictors of susceptibility were not investigated. Here, we found that higher MHC H_E , but not neutral H_E , predicted lower *Bd* prevalence (Tables S7 and S8), consistent with other *Bd* studies that find genetic associations with *Bd* prevalence but not intensity (e.g., Savage et al. 2015; Horner et al. 2017). We also found that individuals homozygous for supertype 4 had a significantly elevated risk of *Bd* infection, while individuals with allele *Rapi**03 had reduced risk of *Bd* infection that was nearly significant. Furthermore, of the two sampled populations with no MHC variability (DOCM and WS, each fixed for a supertype 4 allele), one had 100% *Bd* prevalence and the other had *Bd* positive individuals (Fig. 1). Finally, *Rapi**32 and *Rapi**33 (the only supertype 1 alleles; Fig. 2) form a distinct, well-supported clade with two closely related MHC alleles from *Rana catesbeiana* (Fig. S4), a species with notoriously high tolerance to *Bd* infection (Garner et al. 2006). *Rapi**32 and *Rapi**33 were also the most widespread alleles we recovered, each occurring in the same six populations throughout North America. These patterns imply that supertype 1 may promote *Bd* tolerance, but further sampling of this supertype during cooler months (when *Bd* infections spike) is necessary to confirm this pattern. Overall, we suggest that MHC variation contributes to disease risk in *R. pipiens*, and that our focal MHC locus is evolving at least partially due to disease pressure.

MHC codon evolution in *R. pipiens* provides further evidence that selection acts to promote disease adaptation. We detected positive selection acting on five mammalian-defined PBR residues (Brown et al. 1993), consistent with other amphibian immunogenetic studies (Mulder et al. 2017; Savage et al. 2019). Moreover, one of these codons (position 49; Fig. S5) is a P9 pocket residue significantly associated with *Bd* resistance in the tree frog *Litoria verreauxii alpina* (Bataille et al. 2015) and is the same residue under positive selection in allele Q, an allele significantly associated with *Bd* resistance in *R. yavapaiensis* (Savage and Zamudio 2011, 2016). Additionally, allele *Rapi**03, which is present in the stable eastern populations (Hoffman et al. 2004) and the thriving Arizona metapopulation (O'Donnell et al. 2017), across all years we sampled (1971–2018), and which is associated with reduced risk of *Bd* infection, shares the same residue at position 49 as allele Q. Taken together, these studies suggest *Bd* as a selective agent driving molecular evolution of the focal MHC locus.

Our data support the recent hypothesis that MHC-based tolerance against *Bd* via introgression from eastern

populations may explain the success of the thriving Arizona *R. pipiens* metapopulation (O'Donnell et al. 2017). In the west, allele *Rapi**03 was only recovered in the Arizona metapopulation. *Rapi**03 is the only allele found at both time points for all temporally sampled populations, is associated with a reduced risk of *Bd* infection, and shares functionally important similarities with other *Bd* resistance alleles (see above). While this pattern is consistent with introgression of *Rapi**03 from the east conferring higher survival in the Arizona metapopulation compared to other western populations, explicit evidence of introgression and experimental validation of the relationship between *Rapi**03 and *Bd* resistance are needed. Eventually, such studies could validate the utility of implementing genetic rescue to restore declining western populations (reviewed in Ralls et al. 2020).

Multiple types of pathogen-mediated positive selection likely shape and maintain MHC diversity over the evolutionary history of a species (Takahata and Nei 1990). Determining and distinguishing among these modes of selection in natural populations remain challenging given the required breadth of spatial and temporal sampling (Spurgin and Richardson 2010; Radwan et al. 2020). Our study thus provides a unique opportunity to distinguish among the potential modes of *Bd*-mediated selection—heterozygote advantage, negative frequency-dependent selection, and fluctuating selection—that may be shaping MHC diversity in *R. pipiens*. We found no evidence for heterozygote advantage because MHC heterozygotes were just as likely to be infected with *Bd* as homozygotes. Although no concrete evidence supports negative frequency-dependent selection, it remains plausible based on our temporal comparisons, where many common alleles decreased in frequency over time (Fig. 5; e.g., *Rapi**01 and *Rapi**05) and many rare alleles increased (e.g., *Rapi**07 increased in three populations). However, we do not have temporal Tajima's *D* estimates for neutral loci so cannot interpret the role of selection in shaping MHC-based Tajima's *D* changes over time. Finally, three lines of evidence suggest fluctuating selection may contribute to MHC diversity: (1) environmental variation combined with MHC H_E (but not neutral H_E) was a significant predictor of *Bd* prevalence in most of the top models, (2) pairwise F_{ST} was consistently higher and more variable for MHC compared to microsatellite loci, and (3) the majority of MHC alleles (20/37) were private alleles, and N_E was not correlated to the number of MHC alleles or private alleles. Thus, unique disease dynamics within populations and environmental variation may favor different MHC alleles over time and across populations of varying sizes, contributing to high overall MHC diversity and some differentiation.

Conclusions

In summary, genetic diversity and differentiation at an expressed MHC class IIB locus differ significantly from neutral markers in *R. pipiens* across both spatial and temporal scales. Across sampled populations, we found that higher MHC H_E predicted lower *Bd* prevalence, whereas neutral heterozygosity showed no relationship. MHC supertype 4 conferred a significantly elevated risk of *Bd* infection. In contrast, allele *Rapi*03* was associated with lower *Bd* risk, and Arizona populations of *R. pipiens* may be doing well in the face of *Bd* due to the unique presence of *Rapi*03*, which is absent from all other western sites. Comparing modes of pathogen-mediated selection acting on MHC, we found evidence consistent with fluctuating selection and negative frequency-dependent selection, but no support for heterozygote advantage. Because disease can decimate population genetic diversity across both neutral and functional loci (O'Brien and Evermann 1988), establishing that MHC diversity fluctuations are distinct from overall genetic diversity loss is important for illustrating that frog populations are adapting immunogenetically to disease and other selective pressures.

Data availability

All new genetic data generated for this study has been submitted to GenBank (Accession Numbers: MN984182–984218). All other data used in this study are provided in the Supporting Information.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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