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ORIGINAL ARTICLE

Habitat fragmentation in the Brazilian Atlantic Forest is associated with erosion of frog immunogenetic diversity and increased fungal infections

Anat M. Belasen[1] · Kevin R. Amses[1] · Rebecca A. Clemons[1] · C. Guilherme Becker[2] · L. Felipe Toledo[3] · Timothy Y. James[1]

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Abstract

Habitat fragmentation and infectious diseases threaten wildlife globally, but the interactions of these threats are poorly understood. For instance, while habitat fragmentation can impact genetic diversity at neutral loci, the impacts on disease-relevant loci are less well-studied. We examined the effects of habitat fragmentation in Brazil's Atlantic Forest on amphibian genetic diversity at an immune locus related to antigen presentation and detection (MHC IIB Exon 2). We used a custom high-throughput assay to sequence a fragment of MHC IIB and quantified *Batrachochytrium dendrobatidis* (*Bd*) infections in six frog species in two Atlantic Forest regions. Habitat fragmentation was associated with genetic erosion at MHC IIB Exon 2. This erosion was most severe in forest specialists. Significant *Bd* infections were detected only in one Atlantic Forest region, potentially due to relatively higher elevation. In this region, forest specialists showed an increase in both *Bd* prevalence and infection loads in fragmented habitats. Reduced population-level MHC IIB diversity was associated with increased *Bd* infection risk. On the individual level, MHC IIB heterozygotes exhibited a trend toward reduced *Bd* infection risk, although this was marginally non-significant. Our results suggest that habitat fragmentation increases *Bd* infection susceptibility in amphibians, mediated at least in part through erosion of immunogenetic diversity. Our findings have implications for management of fragmented populations in the face of emerging infectious diseases.

Keywords Amphibians · Habitat fragmentation · Immunogenetics · Brazil's Atlantic Forest · Disease susceptibility · *Batrachochytrium dendrobatidis*

Introduction

Amphibians are in decline worldwide due to stressors including emerging infectious diseases and intensive habitat modification (Stuart et al. 2007; Becker et al. 2010; Scheele et al. 2019). The recent global rise in chytridiomycosis caused by the pathogen *Batrachochytrium dendrobatidis* (*Bd*) has raised questions about whether pathogen virulence and/or amphibian susceptibility has recently increased. Given mounting evidence that *Bd*'s presence predates known declines in several areas of the world (Rodriguez et al. 2014; Talley et al. 2015; Carvalho et al. 2017) and that enzootic lineages of *Bd* can still exhibit high virulence in naïve hosts (Fu and Waldman 2019), increased host susceptibility seems a likely explanation for the rise in disease outbreaks in many regions. One hypothesis is that amphibian susceptibility has increased due to the negative impacts of widespread habitat degradation. Intensive human land-use change that incurs habitat destruction and fragmentation negatively impacts amphibians via several mechanisms. For example, loss of genetic diversity in fragmented populations can reduce population-level fitness and resilience (Allentoft and O'Brien 2010) and increase disease susceptibility (Pearman and Garner 2005). Collectively, the impacts of increasingly intensified land use may have surpassed a

✉ Anat M. Belasen
abelasen@utexas.edu

[1] Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI, USA

[2] Department of Biology, The Pennsylvania State University, University Park, PA, USA

[3] Laboratório de História Natural de Anfíbios Brasileiros, Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, Brazil

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threshold, tipping previously stable populations to a point of increased susceptibility to disease and other stressors, and giving rise to global increases in amphibian disease.

Habitat fragmentation can reduce genetic diversity in surviving wildlife populations (Lesbarrères et al. 2002; Andersen et al. 2004; Johansson et al. 2007; Frankham et al. 2002) or impact selection on immunogenes in the major histocompatibility complex (MHC) that contribute to fitness and immune function (e.g., via changes in local environments and pathogen/parasite communities; Hernandez-Gomez et al. 2019; Gonzalez-Quevedo et al. 2016; Belasen et al. 2019). MHC is composed of two classes, with Class I genes primarily involved in the response to intracellular pathogens and Class II genes primarily involved in the response to extracellular pathogens (Bevan 1987). In particular, MHC Class IIB Exon 2 is associated with conformation of the peptide-binding regions of MHC Class II molecules (Tong et al. 2006), which present pathogen-derived antigen peptides to immune cells to stimulate the adaptive immune response (Bevan 1987; Richmond et al. 2009). Previous studies have shown that MHC IIB genotype is associated with variability in amphibian susceptibility to a variety of pathogens and parasites (Bataille et al. 2015; Savage and Zamudio 2011, 2016; Mulder et al. 2017; Savage et al. 2019; Hernández-Gómez et al. 2019; Belasen et al. 2019) and that MHC IIB heterozygosity confers elevated protection (i.e., heterozygote advantage) against *Bd* (Savage and Zamudio 2011).

Studies of the relationship between habitat fragmentation and MHC diversity have shown mixed results. In general, selection by pathogens is hypothesized to maintain MHC diversity in natural populations through two mechanisms of balancing selection, negative frequency dependent selection (i.e., favoring of rare pathogen resistance alleles), and heterozygote advantage (also referred to as overdominance; Piertney and Oliver 2006). Indeed, in a classic study of MHC diversity, Aguilar et al. (2004) showed that strong balancing selection can maintain high MHC diversity even in the presence of genome-wide genetic erosion (i.e., genetic diversity loss) in historically fragmented vertebrate populations. However, in some taxa, MHC diversity can be naturally low or track neutral genetic diversity. In these cases, demographic factors and genetic drift may outweigh selection (reviewed in Radwan et al. 2009). For example, genetic erosion at MHC IIB was observed in frog populations that had been fragmented and isolated for 12,000–20,000 years on land-bridge islands in southeastern Brazil (Belasen et al. 2019). It remains unclear whether recent (anthropogenic) habitat fragmentation has similarly eroded MHC IIB diversity in amphibians through inbreeding and genetic drift or altered selection. In recently fragmented populations where inbreeding and strong genetic drift are intense enough to outweigh balancing selection, genetic erosion may be expected at MHC loci. This could affect susceptibility to infections, a result of decreased heterozygosity, and/or the loss of disease resistance-associated alleles.

Previous studies of the relationship between habitat fragmentation and *Bd* have not supported an increase in amphibian disease susceptibility with fragmentation. In a meta-analysis, Becker and Zamudio (2011) found that *Bd* prevalence was higher in populations living in unfragmented forested habitats around the world. A logical explanation for this pattern is that *Bd* is a psychrophilic and aquatic fungus, meaning that *Bd* grows optimally in the cooler and wetter environments found in pristine forests (Puschendorf et al. 2009). Nonetheless, *Bd* distribution often does not match habitat suitability model predictions (James et al. 2015). In addition, the majority of studies supporting a negative relationship between *Bd* prevalence and habitat fragmentation focus on individual host species that are locally abundant habitat generalists (Becker and Zamudio 2011; Puschendorf et al. 2009; Kriger et al. 2007). These species may exhibit recalcitrance to both abiotic and biotic stressors (i.e., both fragmentation and *Bd*). In contrast, species that are sensitive to environmental changes or those that exhibit specialized habitat use may experience negative effects of habitat fragmentation more strongly (reviewed in Harrison and Bruna 2012). Thus, it is important to consider a diversity of species to fully understand the impacts of habitat fragmentation on disease susceptibility in diverse tropical systems.

In this study, we examined the effects of recent habitat fragmentation on MHC IIB diversity and *Bd* infection susceptibility in frogs of Brazil's extensively fragmented Atlantic Forest. Typically in population genetic studies, multiple replicate populations of a single focal species would be sampled within each habitat type. However, we were unable to sample multiple populations within a species due to the patchy distribution of populations among small forest fragments. To overcome this limitation in intraspecific replication, we instead sampled two populations of each of six endemic Atlantic Forest species for genetic analyses. Based on observations of habitat use and ability to occupy degraded habitat (Haddad et al. 2013), three of these species are classified as sensitive forest specialists, and three are classified as hardy habitat generalists. Thus, we use species as replicates within each of these two ecological classifications to evaluate whether the impacts of fragmentation vary due to species ecology. The twelve study populations were previously genotyped using restriction site-associated DNA sequencing (ddRAD; Belasen et al. *unpubl.*). We used tissue samples and skin swabs collected from our focal populations to quantify immunogenetic diversity at the MHC IIB locus and *Bd* infection prevalence and load. We also quantified *Bd* infections in three additional species (two habitat generalists and one forest specialist) to improve our detection of community-level patterns in *Bd* (i.e., reduce the potential

for ascertainment bias due to small sample sizes of focal species). We used these combined datasets to address the following research questions: (i) How is MHC IIB diversity and allelic composition affected by fragmentation in forest specialists vs. habitat generalists? (ii) Does fragmentation increase infection susceptibility across a range of species ecologies? (iii) Does MHC IIB diversity and/or genotype determine infection susceptibility?

Methods

Study system and sample collection

Brazil's Atlantic Forest (BAF) is one of the most fragmented tropical ecosystems in the world. Anthropogenic deforestation and fragmentation following European colonization of Brazil have reduced BAF to ~ 13% of its original area, distributed among tens of thousands of small isolated patches more than 80% of which are less than 50 hectares in area (Ribeiro et al. 2009). Yet, the Atlantic Forest remains one of the most biodiverse regions in the world. Amphibian diversity is particularly high in this region, with ~ 660 described species, more than half of which are endemic (Haddad et al. 2013).

We sampled two regions in BAF that contained large tracts of continuous forest as well as small ~ 100-year-old isolated forest fragments within cattle pasture matrix: northeastern São Paulo (January–February 2016, January 2017) and southeastern Bahia (January–February 2017; Fig. 1). In São Paulo, we sampled three forest fragments (0.17–1.83 km^2 in area) in the municipality of São Luiz do Paraitinga (23°09′S 45°15′W, 840 m asl). We also sampled a section of intact continuous forest that has been preserved adjacent to a protected area (Núcleo Santa Virginia, Parque Estadual da Serra do Mar; 23°25′S, 45°11′W, 620 m asl, ~ 17,000 ha total area of intact forest) ~ 30 km from the fragmented area. Similarly, in Bahia, we sampled a forest fragment (0.42 km^2 in area) in the municipality of Igrapiúna (13° 50′S, 39° 13′W, 237m asl). We also sampled a continuous forested site within the Reserva Ecológica Michelin (13° 50′ S, 39° 14′ W, 137m asl, ~

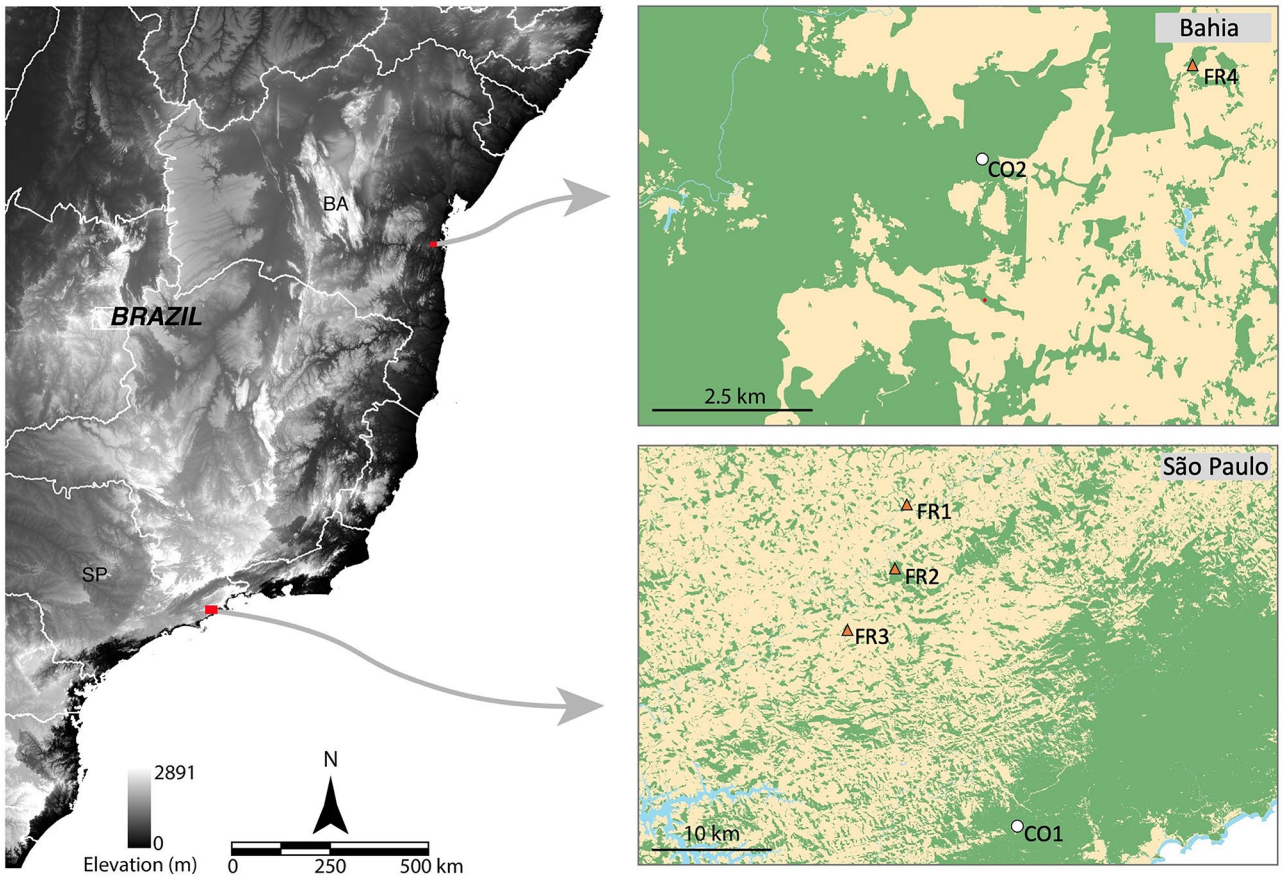

Fig. 1 Sampling locations. Preserved continuous forests (CO1 and CO2) are denoted with white circles, and forest fragments (FR1-FR4) are denoted with red triangles. For each of the six focal species for genetic analyses, two populations were sampled, one from a continuous site and one from a fragmented site. See Table S2 for sample sizes and species associated with each site

1,800 ha total area of natural forest) ~ 2 km from the fragmented area.

We collected tissue and skin swab samples from six focal species sampled from fragmented and continuous habitats in the two regions, including habitat specialists (those that live and reproduce only in forest) and generalists (those that disperse through and reproduce in a variety of habitats including intensively managed agricultural matrix). Four of the focal species were sampled in São Paulo: two forest specialists (Hylidae (*Aplastodiscus leucopygius*) and Brachycephalidae (*Ischnocnema henselii*)) and two habitat generalists (Hylidae: *Dendropsophus minutus* and *Boana polytaenia*). The remaining two focal species were sampled in Bahia: one forest specialist (Hylidae: *Boana semilineata*) and one habitat generalist (Hylidae: *Dendropsophus branneri*). We sampled two populations per species: one population within continuous intact forest and a second population within a forest fragment. To increase power to detect differences in *Bd* prevalence and load, we also collected swab samples from three additional species from the same sampling sites in São Paulo (Odontophrynidae, *Proceratophrys boiei*, forest specialist; Bufonidae, *Rhinella icterica*; Hylidae, *Scinax fuscovarius*; both generalists).

We individually captured frogs using sterile plastic bags and transported to a central field laboratory. Following a ventral rinse with sterilized distilled water, we collected skin swab samples according to a standard protocol (Hyatt et al. 2007) and collected either liver (post-euthanasia) or toe tissue samples for immunogenetic analysis (IACUC protocols for humane animal care and use PRO00005605 and PRO00007691). Euthanized frogs were formalin-fixed and deposited as voucher specimens in the Museu de Zoologia "prof. Adão José Cardoso" (ZUEC), Universidade Estadual de Campinas, São Paulo (Appendix I), while non-lethally sampled frogs were released at the same site they were captured. We extracted DNA using a DNeasy kit (Qiagen) with a modified protocol for swabs and the manufacturer's protocol for tissues.

MHC IIB sequencing

We amplified an ~ 200 bp fragment of MHCIIB Exon 2, an immunogenetic locus previously shown to associate with Bd susceptibility (Bataille et al. 2015; Savage and Zamudio 2011), from tissue DNA extracts with amphibian MHC IIB primers BCF6 and BobomSR (May and Beebee 2009). These primers were designed based on MHC IIB Exon 2 sequences from *Bombina bombina* and *Bufo calamita* and were subsequently used to amplify this relatively conserved locus in a broad diversity of anuran species (Bataille et al. 2015; Belasen et al. 2019). Prior to additional library prep and sequencing, we cloned and sequenced a subset of PCR products using a TOPO TA cloning kit (Invitrogen) and blue/white screening. Successful clones were sequenced and compared against the NCBI GenBank database using blastx to confirm homology to amphibian MHC IIB Exon 2. In two focal species (*D. minutus* and *A. leucopygius*), clean homologous sequences could not be consistently produced using BCF6 and BobomSR, likely due to spurious amplification of paralogs. We designed species-specific primers for these two species using a genome walking approach (Clontech Universal Genome Walker Kit 2.0) to design new primers that would amplify only orthologous loci (~400 bp of exon and a portion of flanking intron) from samples. Samples were PCR-amplified using primers modified with an attached indexing primer overhang (Table S1) and then pooled in equimolar volumes, purified, and sequenced on the Illumina MiSeq platform (250 bp paired-end nano run) at the University of Michigan Microbial Systems Molecular Biology Laboratory (Supplemental Methods).

MHC IIB genotyping and supertyping

Demultiplexed MHC IIB sequence reads were bioinformatically processed using the Mothur MiSeq pipeline, and alleles were clustered into ≥ 99% identical "OTUs" (operational taxonomic units) that represent putative MHC IIB Exon 2 alleles (Kozich et al. 2013; Supplemental Methods). To confirm that haplotypes were orthologous, we constructed a PhyML tree using the HKY85 model and 100 bootstraps in Seaview (vrs. 4.5.4; Gascuel 1997; Gouy et al. 2010). To identify positively selected sites (PSS) and MHC IIB supertypes, allele sequences were translated into amino acid sequences in MEGA (vrs. 7.0.26-mac). Sequences were aligned with a frog MHC IIB dataset (Bataille et al. 2015) to identify amino acid residues in analogous positions to human MHC class II alleles associated with peptide binding region (PBR) pocket conformation (PBR groove pockets 4, 6, 7, and 9; Bataille et al. 2015; Mulder et al. 2017; Brown et al. 1993; Tong et al. 2006). PSS in the amino acid alignment were identified using a fixed effects likelihood model of site selection implemented in Datamonkey 2.0 (Weaver et al. 2018; Pond and Frost 2005). We clustered alleles into functional supertypes based on PSS amino acid physicochemical properties (amino acid z-descriptors z1-z5; Sandberg et al. 1998) using a BIC-based k-means clustering algorithm and discriminant analysis of principle components (DAPC) implemented in adegenet in R (Jombart et al. 2010).

Population genetic analyses

To determine whether MHC haplotypes clustered according to species relatedness or local habitat, we constructed and

visualized a haplotype network using the pegas package in R (vrs. 3.5.1; R Core Team 2018; Paradis 2010). The network was constrained to four focal species comprising two congeneric pairs: *D. minutus* and *B. polytaenia* from São Paulo and *D. branneri* and *B. semilineata* from Bahia.

To determine the impacts of fragmentation on MHC IIB diversity, we calculated summary statistics in DnaSP (Librado and Rozas 2009). These included allelic diversity (N_A), observed and expected heterozygosity (H_O and H_E), and nucleotide diversity (π). To evaluate whether fragmentation was associated with significant reductions in immunogenetic diversity, 95% confidence intervals were calculated for mean H_E and π for each population. MHC IIB genetic structure was evaluated among populations within each species by calculating the fixation index (F_{ST}) in R. To compare MHC IIB diversity to neutral genetic diversity, we ran separate general linear models in which MHC IIB diversity summary statistics H_O, H_E, and π were treated as dependent variables, and the analogous summary statistic from a reduced representation genomic library (ddRAD) constructed from the same samples was treated as a fixed effect independent variable. Additional models that included habitat type and species ecology (generalist vs. specialist) as factors were constructed using a stepwise additive model building procedure. Adjusted R^2 values were used to select the best model for each MHC IIB summary statistic. To test for signatures of selection across MHC IIB Exon 2, we calculated the ratio of non-synonymous to synonymous sites (dN/dS) for each population and statistically analyzed the difference between dN and dS using *z*-tests in MEGA (vrs. 7.0.26-mac). To test for evidence of population bottlenecks, we calculated Tajima's D (Tajima 1989) in MEGA.

Detection and analysis of Bd infections

We analyzed swabs using a standard qPCR assay (Boyle et al. 2004). Standard curves were produced using seven serial dilutions (10^6-10^0 zoospore equivalents, hereafter ZE) of CLFT035, a *Bd*-GPL culture isolated from a BAF tadpole. Samples were run in duplicate to ensure accurate quantification, and only those that amplified ≥ 1 ZE were considered positive for *Bd.* Samples that amplified in only one replicate were re-run and were considered positive if at least 2/4 replicates amplified ≥ 1 ZE. Mean load in ZE was recorded for each positive sample. For São Paulo samples, we only processed and analyzed *Bd* swabs collected during the 2016 sampling season to avoid confounding year-to-year differences, and because only a small number of samples were collected from São Paulo in 2017.

We compared *Bd* infection rates across species ecologies (specialist vs. generalist) and habitat types (fragmented vs. continuous) using chi-square tests computed in R. *Bd* loads were log-transformed and compared across species ecologies and habitats using a non-parametric two-factor Scheirer-Ray-Hare test, and across species ecologies only using a Wilcoxon test for non-normally distributed data. To examine the relationship between genetic diversity and infections, we constructed general linear models in R with *Bd* load as the dependent variable and additive stepwise combinations of four explanatory variables: ddRAD or MHC IIB genetic diversity, species identity, species ecology, and habitat type. The model with the highest adjusted R^2 values was selected as the best model for each measure of genetic diversity. We used *t*-tests to determine whether *Bd* loads were associated with MHC IIB heterozygosity on an individual-level and chi-square tests to compare *Bd* infection rates between MHC IIB heterozygotes and homozygotes for both allelic genotype and supertype.

Results

Immunogenetic diversity

We recovered 72 unique MHC IIB haplotypes across the six focal species. Construction of a haplotype network showed that haplotypes tend to cluster by genus rather than by sampling area (Fig. 2). A single trans-specific haplotype was observed (i.e., the same haplotype occurred in different species). While most haplotypes clustered within genera, the trans-specific haplotype was shared between *D. branneri* and *B. semilineata* (both Bahia species; haplotype XL), and one *D. branneri*-specific haplotype (haplotype XLI) clustered with *Boana* (from São Paulo and Bahia) haplotypes on the network. Haplotypes also predominantly clustered by species in the maximum likelihood tree (Fig. S1).

Five codon positions across the MHC IIB alignment exhibited strong positive selection (dN/dS > 10) and aligned with putative PBR pocket residues (Fig. S2). The 72 haplotypes condensed into seven unique MHC IIB supertypes that overlapped across regions and species (Figs. S1 and S3). Two supertypes were found only in a single species: ST1 was found only in *D. branneri* (Bahia), and ST6 was found only in *D. minutus* (São Paulo).

MHC IIB expected heterozygosity (H_E) was significantly lower in fragmented populations relative to continuous populations in 5/6 focal species (Fig. 3A and Table S2). MHC IIB nucleotide diversity (π) was also lower in fragmented

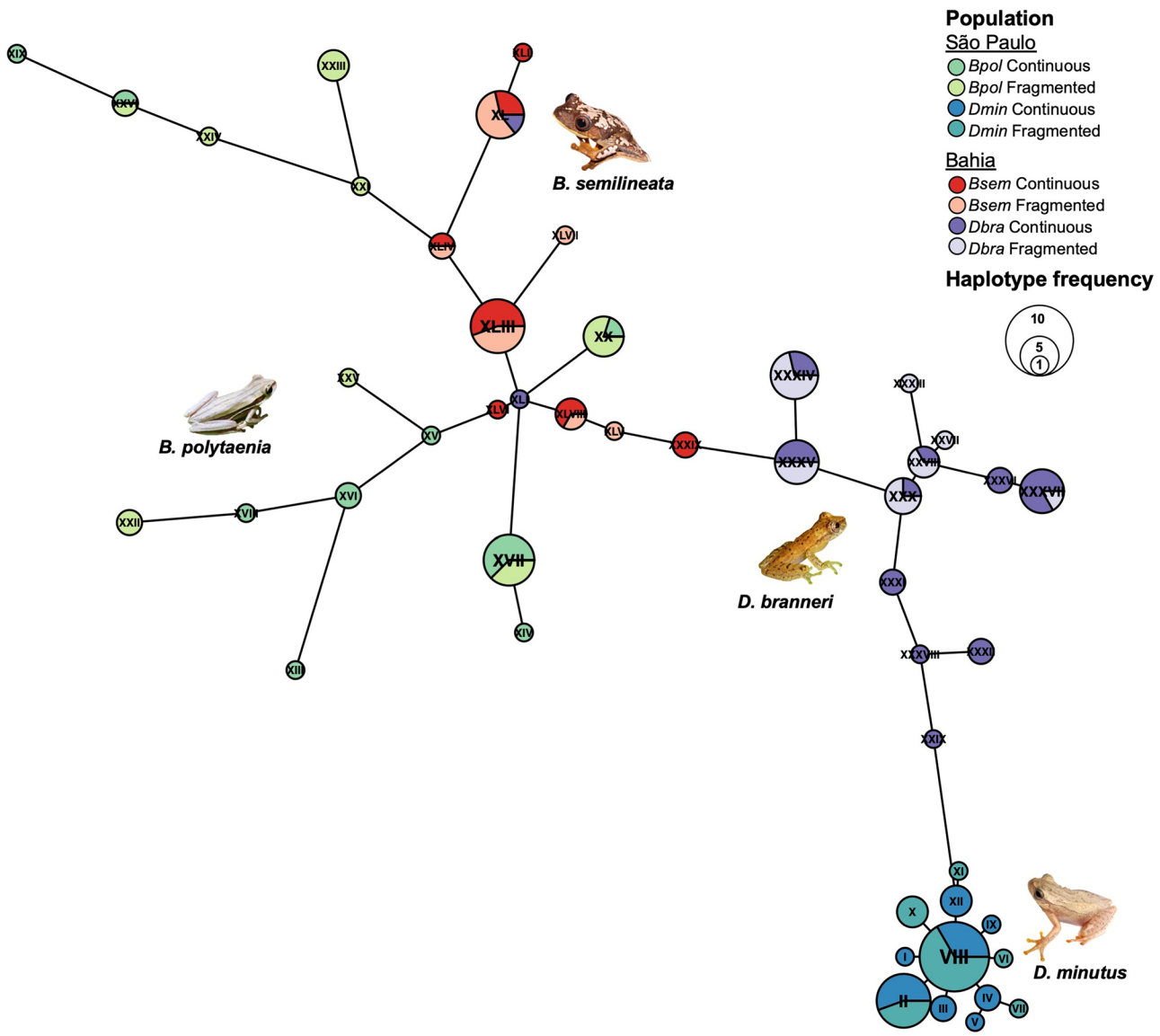

Fig. 2 MHC IIB haplotype network for four focal species. Circle size is proportional to haplotype frequency, colors correspond to the populations in which each haplotype is found, and the length of the links between haplotype circles correspond to the genetic distance between haplotypes. XL was the only haplotype found in more than one species (*B. semilineata* and *D. branneri*). Photos by A. M. Belasen and T. Y. James

populations in all three specialist species and in the generalist *D. branneri* (Fig. 3B and Table S2).

According to dN-dS z-tests, significant signatures of selection on MHC IIB were found only in the São Paulo specialists *A. leucopygius* (positive selection in both populations) and *I. henselii* (negative in fragmented population and positive in continuous population; Table S2). No populations showed signatures of population bottlenecks according to Tajima's D (Table S2).

Relative to genetic differentiation (F_{ST}) across ddRAD loci, MHC IIB showed greater genetic differentiation in three species (*A. leucopygius*, *D. minutus*, and *D. branneri*) and lower genetic differentiation in the remaining three species (*I. henselii*, *B. semilineata*, and *B. polytaenia*; Fig. 3C). When MHC IIB diversity summary statistics H_O, H_E, and π were compared with summary statistics generated from ddRAD data, a significant association was only found for H_O, with a negative relationship between

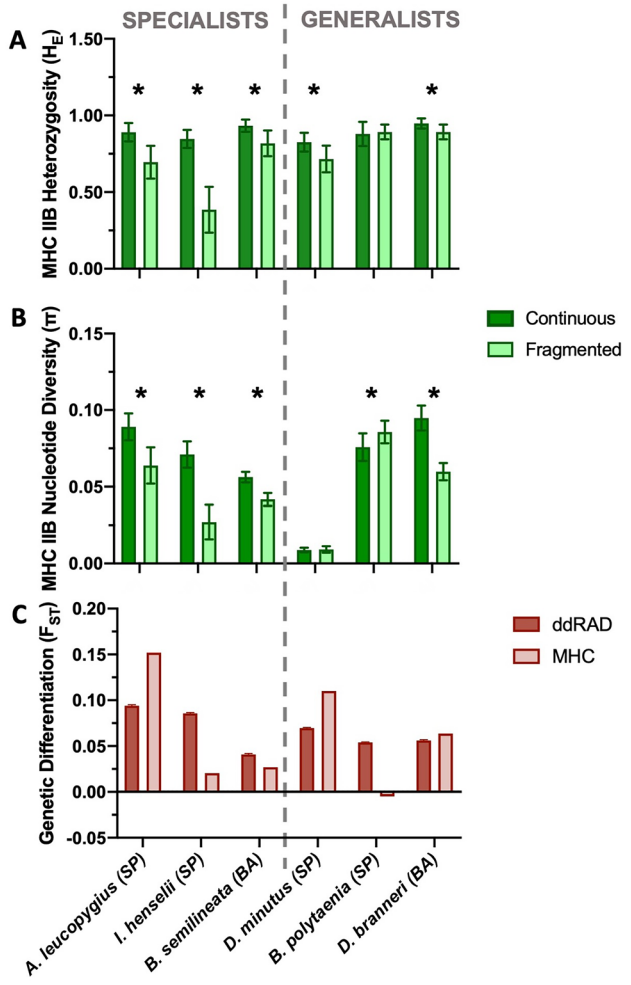

Fig. 3 MHC IIB summary statistics across all focal species. Sampling region (SP = São Paulo, BA = Bahia) is specified in parentheses after each species' name. **A,B** MHC IIB immunogenetic diversity erodes in fragmented populations as measured by both expected heterozygosity (**A**) and nucleotide diversity (**B**). Dark green bars represent populations from continuous forests, and light green bars represent populations from fragmented forests. Asterisks represent a significant difference according 95% confidence Intervals shown by error bars. **C** Genetic differentiation (fixation index, F_{ST}) at MHC IIB vs. ddRAD markers. ddRAD F_{ST} mean values are shown by red bars with 95% CI error bars, and MHC IIB F_{ST} values are shown by pink bars

MHC IIB and ddRAD H_O (SLR, β = -3.7, $p = 0.008$, $R^2 = 0.45$; Fig. S4).

Incidence of Bd infections

Bd infections were detected in all sites sampled in São Paulo. After running a subset of samples (~50) across both species and study sites from lowland Bahia, we found ~ 5% prevalence of *Bd* with positive samples showing very low loads (~ 1 ZE). As this is consistent with previous findings of very low *Bd* prevalence and loads from lowland areas in the Atlantic Forest (Lambertini et al. 2021; Gründler et al. 2012), and as loads < 100 typically do not result in disease (Kinney et al. 2011), we restricted analyses of *Bd* infections to São Paulo populations.

Within São Paulo, *Bd* infection rates were higher in fragmented populations relative to continuous populations (26.7% mean *Bd* prevalence in fragmented populations compared with 10% in continuous populations; $X^2(1) = 10.22$, $p < 0.01$; Table S3 and Fig. 4A), and in specialists relative to generalists (34.2% mean *Bd* prevalence in specialists compared with 13.3% in generalists; $X^2(1) = 9.70$, $p < 0.01$; Table S3 and Fig. 4A). A full factorial test of *Bd* infection loads across habitat types and species ecologies was non-significant (Scheirer-Ray-Hare test on log-transformed *Bd* loads; habitat type, $H(1) = 2.33$, $p = 0.13$; species ecology, $H(1) = 2.72$, $p = 0.09$; habitat type*species ecology interaction, $H(1) = 0.37$, $p = 0.54$). When habitat type was removed from the analysis, the effect of species ecology on log *Bd* load *Bd* load was statistically significant, with higher loads on average in specialists ($W(1) = 92$, $p < 0.05$, Fig. 4B).

There was a significant negative relationship between *Bd* prevalence and population-level MHC IIB diversity for both measures of heterozygosity. The best models included habitat type (fragmented vs. continuous) as an explanatory variable (MHC IIB H_E, β = -84.61, $p = 0.03$, overall model $p = 0.016$, $R^2 = 0.88$; MHC IIB H_O, β = -52.64, $p = 0.031$, overall model $p = 0.026$, $R^2 = 0.84$; Fig. S4). There were no significant relationships between *Bd* prevalence and MHC IIB nucleotide diversity or any measures of neutral genetic diversity (ddRAD data; GLMs, $p > 0.05$).

On the individual level, MHC IIB heterozygotes tended to be less infected with *Bd* relative to homozygotes, although this was marginally non-significant (X^2 (1) = 3.04, $p = 0.08$; Fig. 4C). Supertypes that were shared across São Paulo species (ST2, ST4, ST5, and ST7) did not show significant associations with *Bd* infections (X^2 tests, $p > 0.1$ for all supertypes). There was also no relationship between individual-level MHC IIB allelic genotype (heterozygote vs. homozygote) or supertype (one vs. two supertypes per individual) and *Bd* load (Mann-Whitney *U* tests on log-transformed *Bd* load, $p > 0.1$).

Discussion

Habitat fragmentation is associated with erosion of immunogenetic diversity

In this study, we built upon previous studies of amphibian immunogenetics and infection risk to quantify the effects of landscape modification on MHC IIB diversity and infection susceptibility across ecologically divergent host species in the wild. We found that populations in fragments

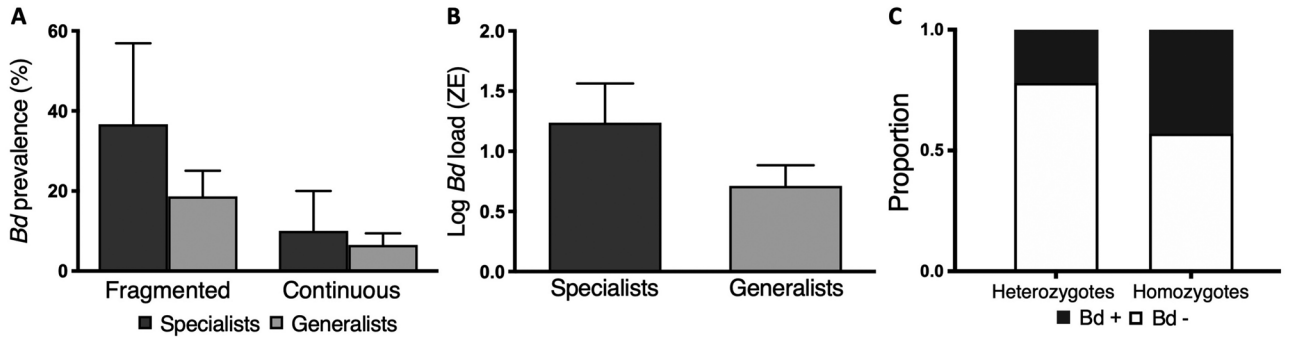

Fig. 4 *Bd* incidence across populations and MHC IIB genotypes. (**A**) and (**B**) include data from six São Paulo amphibian species, while (**C**) only includes data from the four species that were genotyped for MHC IIB (see text for details). **A** *Bd* prevalence in São Paulo across habitat types and species ecologies (forest specialists in dark gray, habitat generalists in light gray). **B** Log-transformed *Bd* infection loads across species ecologies (forest specialists in dark gray, habitat generalists in light gray). **C** Proportion of *Bd*-infected (black) versus uninfected (white) frogs according the MHC IIB allelic genotype

exhibited reduced MHC IIB diversity relative to populations in continuous habitats, with the most severe genetic erosion in the forest specialists *A. leucopygius* and *I. henselii*. We also found that across all species, MHC IIB diversity was inversely related to overall genetic diversity based on ddRAD markers. Taken together with low Tajima's D values and MHC IIB F_{ST} values differing from ddRAD marker F_{ST} values, this suggests that the loss of MHC IIB diversity may not exclusively be due to genetic drift or inbreeding in fragmented populations. In half of our focal species, MHC IIB genetic differentiation was lower than expected based on genome-wide genetic differentiation, suggesting that similar MHC IIB alleles are selected in different populations. This is corroborated by the MHC IIB haplotype network, which does not show clustering according to population.

Trans-specific polymorphism is thought to be common in MHC genes (Klein 1987) and may be especially likely across species that encounter similar pathogens. However, among the 72 MHC IIB haplotypes, we recovered only one haplotype that was shared between focal species (haplotype XL, supertype 2). This haplotype was found in both Bahia species (*B. semilineata* and *D. branneri*) and at moderate relative abundance (7/62 haplotypes, found in 7/31 individuals in Bahia). This suggests that the local environment and/or parasites in Bahia that were not measured in this study could be driving selection for this allele.

The positively selected codons that we detected across the MHC IIB alignment are corroborated by previous studies as sites that impact PBR pocket shape and thus impact pathogen recognition (Bataille et al. 2015; Mulder et al. 2017). However, the diversity of haplotypes and supertypes that we recovered is relatively lower than might be expected based on previous studies. For example, Savage and Zamudio (2016) recovered 84 alleles and 4 supertypes across 8 populations of a single species (128 individuals). In our study, we analyzed sequences from a similar number of individuals ($n = 102$) but included six focal species spanning two families and four genera. It is somewhat surprising that only seven functional supertypes were recovered across this level of species diversity. As MHC IIB supertypes have been characterized in only a small number of amphibian species to date, it is unknown how many supertypes exist across diverse amphibian species. Further interspecific studies of amphibian MHC could aid in determining whether supertypes show a high degree of trans-specific polymorphism in response to similar pathogens or other relevant selective pressures. We also cannot rule out that our molecular methodology could have resulted in an underestimate of immunogenetic diversity in our focal populations. Development of deep sequencing methods for amphibian MHC loci could be beneficial to future research and understanding of functional immunogenetics across species.

Pathogen prevalence and load vary with elevation, habitat fragmentation, and immunogenetics

The relationships we observed between infections, landscape factors, and MHC diversity shed light on community-level disease dynamics in Brazil's Atlantic Forest. *Bd* prevalence and loads were extremely low in the lowland Bahia sampling region. However, *Bd* was detected in all São Paulo populations, with the highest *Bd* prevalence in fragmented populations and in forest specialist species. At the population level, we observed an inverse relationship between MHC IIB diversity and *Bd* prevalence; on the individual-level, MHC IIB heterozygotes were less likely to be infected with *Bd*. This corroborates previous studies in which MHC IIB heterozygotes showed lower *Bd* susceptibility (Savage and Zamudio 2011, 2016). In other studies, the opposite was observed: individuals or populations with higher heterozygosity experienced higher *Bd* infection risk (Addis et al. 2015; Kosch et al. 2016a), potentially due to correlations

between heterozygosity, dispersal, and *Bd* transmission in more genetically diverse populations. Based on these findings, we may expect to detect fewer *Bd* infections in fragmented populations, as limitations on dispersal due to fragmentation could result in reduced pathogen transmission. However, in our study area, we found no evidence of reduced *Bd* transmission, potentially as a result of generalist species transmitting *Bd* from continuous habitats to isolated forest specialist populations. As habitat generalists show moderate prevalence and relatively low *Bd* loads overall, these species could hypothetically serve as tolerant pathogen carriers in this multi-host system.

It is possible that the associations we observed between *Bd* infections and MHC IIB are due to causal relationships between infection susceptibility and immune function, or to both factors being independently associated with fragmentation. For example, MHC IIB selection dynamics may be altered due to proximity to agriculture (Hernández-Gómez et al. 2019) rather than or in addition to selection by parasites in the fragmented landscape. Likewise, infection susceptibility may be increased by fragmentation due to physiological stress (Carey et al. 1999) rather than via impacts on genetic diversity. Nonetheless, a growing number of studies support the role of MHC IIB in *Bd* susceptibility. Both comparative genetic studies across diverse host species and infection experiments have supported the mechanistic relationship between MHC IIB genotype and *Bd* susceptibility (Savage and Zamudio 2011; Bataille et al. 2015). Future studies such as common garden experiments would be valuable in distinguishing between the impacts of immunogenetics versus stress in determining disease susceptibility in fragmented, immunogenetically eroded populations.

While we did not detect relationships between MHC IIB supertype and *Bd* infection risk, we observed interesting patterns in MHC IIB haplotype and supertype distribution with potential relevance to pathogens other than *Bd*. For instance, haplotypes from the two Bahia species (*B. semilineata and D. branneri*) show high connectance and clustering on the haplotype network, which may belie selection by pathogens found in Bahia but not São Paulo. Similarly, supertype 1 is only found in *D. branneri*, while supertype 6 is only found in *D. minutus*; these supertypes may play roles in defending against pathogens specific to these two species, which are both considered relatively hardy and are often observed in degraded edge habitats where pathogen/parasite spillover may be more common (Borremans et al. 2019). While the majority of recent work on associations between MHC and infections in amphibians has focused on *Bd*, susceptibility to infection by a diversity of pathogens and parasites may also be associated with MHC diversity (Belasen et al. 2019). Future amphibian immunogenetics studies should expand beyond *Bd* to clarify relationships between genetic diversity, geographic region or environment, and species-level disease susceptibility.

Taken together, our results suggest that habitat fragmentation is associated with decreased immunogenetic diversity and increased infections in susceptible amphibians. We detected immunogenetic diversity loss in fragmented amphibian populations that has potentially resulted from inbreeding and genetic drift, although we also detected signatures of selection in some fragmented populations. In addition, we found that habitat fragmentation does not reduce *Bd* incidence. We hypothesize that this generalist pathogen gains access to isolated forest specialist host populations via high-dispersing tolerant habitat generalist hosts. Our study expands knowledge of amphibian immunogenetics by demonstrating that habitat fragmentation can affect MHC IIB genetic diversity, which has important implications for conservation and management of populations that are susceptible to *Bd* and other pathogens. Future studies of amphibian genetic diversity and disease should consider the range of responses across the host community to gain a holistic understanding of wildlife vulnerability in fragmented natural systems.

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Author contribution AMB and TYJ conceived of the project; AMB, CGB, LFT, and TYJ performed the fieldwork; AMB and RAC performed the lab work; AMB and KRA analyzed the data; AMB, KRA, and CGB produced the figures; AMB wrote the paper; all authors contributed to reviewing and revising the paper.

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Data availability The data that support the findings of this study are openly available in the supplementary material and GenBank (ddRAD data: SRA accessions SAMN25158801, SAMN25182090; MHC sequences: GenBank accessions OM362104 - OM362229).

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