



# Long-Term Habitat Fragmentation Is Associated With Reduced MHC IIB Diversity and Increased Infections in Amphibian Hosts

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Habitat fragmentation and wildlife disease are two widespread drivers of biodiversity loss, yet few empirical studies have explored their interactions. In this study, we utilized a naturally fragmented island system to examine the impacts of fragmentation on genetic diversity and amphibian infection dynamics. We determined the impacts of fragmentation on genetic diversity at the immunity locus MHC IIB, a hypothesized predictor of disease susceptibility. Contrary to the expectation that MHC diversity would remain high due to balancing selection, island populations lost genetic diversity at this locus while simultaneously experiencing positive selection at MHC IIB. We then used Next-Generation Sequencing to identify a variety of potential eukaryotic parasites from amphibian skin swabs. Island populations exhibited higher potential parasite richness (proportion of eukaryotic microbe operational taxonomic units or OTUs from parasitic taxa) relative to mainland populations. MHC homozygotes hosted a lower diversity of potential parasites, and population-level MHC diversity was negatively associated with parasite richness. Our results show that genetic erosion can occur at the MHC IIB locus following fragmentation, which may contribute to increased susceptibility to parasites.

**Keywords:** habitat fragmentation, amphibians, parasites, immunogenetics, Brazilian Atlantic Forest

## INTRODUCTION

Habitat loss and fragmentation are arguably the most widespread drivers of biodiversity loss (Perfecto and Vandermeer, 2010). Habitat fragmentation can significantly impact population resilience against stressors including emerging infectious diseases (Dobson and Foufopoulos, 2001). These effects include the loss of genetic diversity over time due to genetic drift compounded by inbreeding (Newmark, 1995; Zuidema et al., 2017), which in turn may increase disease susceptibility (Altizer et al., 2003). Fragmentation may also reduce host contact rates and disease transmission, which could alter selection on disease resistance alleles. This could also result in the loss of genetic diversity if there was strong selection for resistance to a few remaining local pathogens.

Genetically eroded hosts have exhibited increased disease susceptibility both in laboratory experiments (Arkush et al., 2002; Spielman et al., 2004; Ilmonen et al., 2008; Ellison et al., 2012) and

in studies of natural populations (Pearman and Garner, 2005; Roca et al., 2010; Ellison et al., 2011). Low genetic diversity has also been associated with increased levels of parasitism, which suggests that there is a relationship between immune function and genetic diversity (Macdougall-Shackleton et al., 2005) and that genetically eroded individuals may be more susceptible to opportunistic infections (Anaissie, 1992). These associations between inbreeding and disease may be related to loss of resistance alleles at immunogenetic loci—genetic regions that are important in pathogen recognition and immune response.

Immunogenetic studies in vertebrates often focus on the major histocompatibility complex (MHC), a gene family composed of two major classes (Bernatchez and Landry, 2003). Molecules encoded by the MHC bind with antigenic peptides, which they present on the outside of cells in infected tissue to signal T-cells to mount an immune response (Richmond et al., 2009). MHC heterosis (heterozygote advantage) is believed to be common, as heterozygosity is associated with a broader repertoire of MHC molecules and therefore enhanced immunity (Doherty and Zinkernagel, 1975; McClelland et al., 2003; Whitehorn et al., 2011). Indeed, MHC heterozygosity can be exceedingly high relative to other loci (Gaudieri et al., 2000; Landry et al., 2001; Hambuch and Lacey, 2002), even in otherwise genetically impoverished populations (Aguilar et al., 2004). In some cases, however, specific MHC genotypes have been more strongly associated with disease resistance than heterozygosity (Schwensow et al., 2007; Deter et al., 2008).

MHC genotype determines the structure of the peptide binding region of MHC molecules. This structure in turn determines the types of antigen peptides, and essentially the types of pathogens, that can be recognized (Piertney and Oliver, 2006). Alleles encoding MHC molecules that can recognize endemic pathogens are most likely to be common in the local host gene pool. For example, a human HLA (MHC analog) haplotype associated with malaria resistance is common in West Africa, where malaria is endemic, but not elsewhere (Hill et al., 1991). Introduced pathogens are more likely to cause severe outbreaks and declines, which may be because in naïve host populations, particular MHC alleles associated with an effective pathogen-specific immune response are likely to be relatively uncommon. Indeed, long-term parasite release has been hypothesized as a mechanism driving the susceptibility of island endemics to introduced pathogens and parasites (reviewed in Wikelski et al., 2004).

Amphibians serve as models for understanding the interactions of fragmentation, genetics, and disease. Habitat fragmentation is considered one of the most important drivers of amphibian declines globally (Kiesecker et al., 2001; Cushman, 2006). While this may be due to direct effects of fragmentation on amphibian dispersal, amphibians also experience indirect effects including loss of genetic diversity. Many amphibians are especially sensitive to this genetic impoverishment, in part due to naturally small effective population sizes (Allentoft and O'Brien, 2010). Reduced genetic diversity in amphibians has been correlated with decreased growth, survival, and population resilience (Lesbarrères et al., 2005; Arens et al., 2007). Although few studies have tested for correlations between genetic diversity

and disease susceptibility (but see Pearman and Garner, 2005), increased appreciation of the widely variable disease susceptibility within and among amphibian species (Briggs et al., 2010; Gahl et al., 2012; Bielby et al., 2015; Gervasi et al., 2015) has led to increased focus on the relationship between amphibian genetics and disease. Notably, the MHC IIB locus has been shown to modulate susceptibility to chytridiomycosis caused by the fungal skin pathogen *Batrachochytrium dendrobatidis* (*Bd*), with studies suggesting both that specific alleles and heterozygosity can provide an advantage against *Bd* (Savage and Zamudio, 2011; Bataille et al., 2015; Kosch et al., 2016).

The majority of recent amphibian disease research focuses on only a few pathogens, despite widespread evidence that amphibians are vulnerable to a diversity of pathogens (Kiesecker et al., 2001; Briggs et al., 2010). Multiple pathogens and parasites have been implicated in amphibian declines, notably the chytrid fungi *Bd* and *B. salamandrivorans*, the zygomycete fungus *Mucor amphibiorum*, oomycete parasites including *Saprolegnia* spp., Ranaviruses, and metazoans including trematodes (Speare et al., 1994; Martel et al., 2013; Gleason et al., 2014). Amphibians are also known to experience infection by alveolates including *Perkinsea* spp. (Chambouvet et al., 2015), the apicomplexan *Hepatozoon* (Desser et al., 1995), the ichthyosporean *Anurofeca richardsi* (Rowley et al., 2013), the ectoparasitic ciliate *Epistylus* sp. (Pritchett and Sanders, 2007), and a variety of helminth parasites (Dyer, 1991; Yildirimhan et al., 2006). Much attention has focused on the role of *Bd* as an important disease agent in amphibians worldwide, while fewer disease surveys have explored other parasitic taxa.

In this study, we examined the effects of fragmentation on amphibian immunogenetic diversity at the MHC IIB locus and potential susceptibility to a diversity of parasites. The effects of fragmentation are often subject to time lags whereby the impacts of current anthropogenic fragmentation may not be fully realized for several generations (Tilman et al., 1994; Arenas et al., 2014; Mona et al., 2014). Therefore, to examine the long-term impacts of fragmentation on immunogenetic diversity, we utilized a natural laboratory in the Brazilian Atlantic Forest as a model system. The Atlantic Forest contains dozens of land-bridge islands, which were connected to the mainland prior to the Pleistocene (Suguio et al., 2005). These islands are essentially forest patches that were naturally isolated by marine incursions 12,000–20,000 years ago; thus they represent very old habitat fragments. Remaining frog populations were once part of contiguous mainland populations, and are now isolated to the islands (Bell et al., 2012; Duryea et al., 2015).

We sampled populations of a single frog species (Cycloramphidae: *Thoropa taophora*) that is widespread across the Atlantic Forest coast. We collected tissue and skin swab samples to respectively analyze frog MHC IIB diversity and parasite diversity using DNA sequencing. These data allowed us to test the following predictions: (i) immunogenetic diversity at the MHC IIB locus is maintained despite overall losses of neutral genetic diversity in fragmented (island) populations; (ii) genetically eroded populations exhibit higher parasitism; and (iii) MHC IIB genotype is associated with differences in parasite richness.

## MATERIAL AND METHODS

### Study System and Field Sampling

The study area spans seven land-bridge islands located off of the north coast of São Paulo state in Southeastern Brazil, and four sites found on the nearby coastal mainland (**Figure 1A**). The island and mainland sites are characterized by typical Atlantic rainforest vegetation bordered by rocky coastal areas and exhibit similarly low levels of human perturbation. On the Atlantic Forest land-bridge islands, a small number of amphibian species have maintained stable populations since island formation (Bell et al., 2012). The best-studied of these is *Thoropa taophora*, a Cycloramphid frog that inhabits wet rocks and is widespread along the São Paulo coast (Sazima, 1971; Bokermann, 1974; Giaretta and Facure, 2004). The island *T. taophora* populations were recently shown to possess reduced genetic diversity relative to mainland populations (evaluated with microsatellite loci), with estimated divergence times approximately consistent with island age estimates (ranging from 4,000 to 28,000 years for most island populations), little significant migration among populations, and no effect of island size or distance from mainland on migration or population genetic diversity (Duryea et al., 2015).

Adult *T. taophora* frogs ( $n = 4\text{--}30$  per site, 179 total) were individually sampled from each of the 10 study populations between January and March 2015 (SISBio collection permit 27745-13). Frogs were washed with sterile water and then swabbed using a standard sampling protocol (Hyatt et al., 2007). Toe tissue was collected from each individual with sterilized dissection scissors for analysis of host genetics. DNA was extracted from swab and tissue samples using the Qiagen DNeasy Blood and Tissue kit.

### Immunogenetic Analyses

A ~120 bp fragment of the MHC IIB locus was PCR-amplified from tissue DNA extracts (May and Beebe, 2009; **Supplementary Material**) and sequenced at the University of Michigan Sequencing Core. Haplotypes were predicted using Phase (Version 2.1.1) (Stephens et al., 2001) to obtain allele frequencies, which were used to calculate allelic richness ( $N_A$ ) and observed and expected heterozygosity ( $H_o$  and  $H_e$ ). Segregating sites ( $S$ ) and nucleotide diversity ( $\pi$ ) were calculated in DnaSP (Version 5; Librado and Rozas, 2009).  $T$ -tests were used to evaluate differences in  $S$  and  $\pi$  among populations, and simple linear regression was used to compare MHC  $N_A$  and heterozygosity with microsatellite  $N_A$  and heterozygosity (extracted from Duryea et al., 2015) in R (Version 3.3.0; Lighten et al., 2014).

The ratio of non-synonymous to synonymous nucleotide changes ( $dN/dS$ ) across the MHC alignment and associated  $p$ -values for departure from the neutral expectation ( $dN/dS = 1$ , no selection) were calculated in MEGA (vrs. 6.06-mac; Tamura et al., 2013).  $dN/dS$  was calculated across the entire dataset, and also for each population and for each habitat type (island vs. mainland). Tajima's  $D$  and the  $p$ -value associated with its departure from a neutral expectation ( $D = 0$ , random evolutionary processes; Tajima, 1989) were calculated in DnaSP. To examine evolutionary and geographic patterns across MHC

IIB alleles, an MHC haplotype network was visualized and the fixation index ( $F_{ST}$ ; a measure of population differentiation) was calculated using the pegas package in R (Paradis, 2010). Mean  $F_{ST}$  was calculated for nine microsatellite markers from the same populations (extracted from Duryea et al., 2015). A partial Mantel correlation was used to evaluate associations between MHC  $F_{ST}$  and geographic distance among populations while accounting for neutral (microsatellite)  $F_{ST}$ . MHC  $F_{ST}$  was compared with mean microsatellite  $F_{ST}$  to determine whether similar evolutionary processes were impacting the MHC.

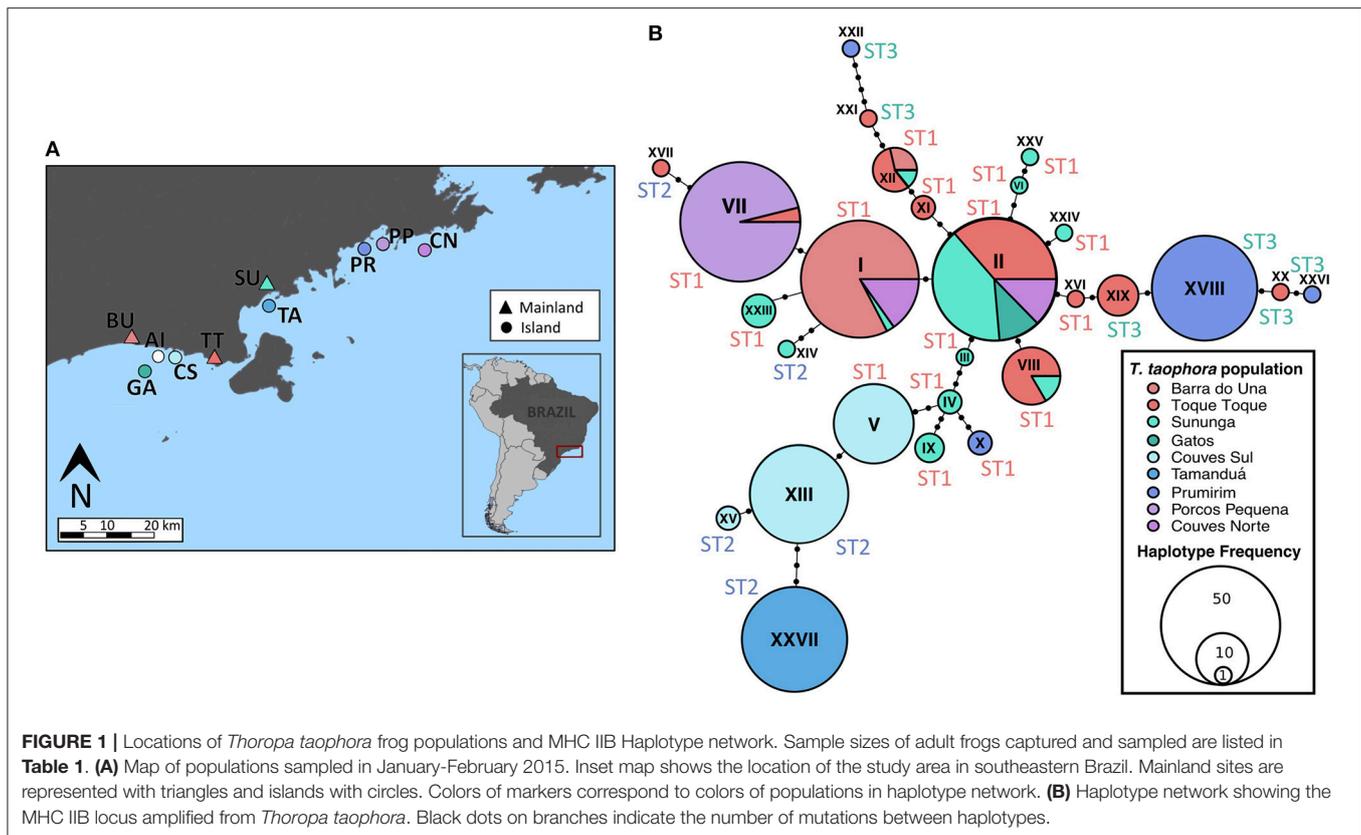
MHC haplotypes were condensed into functionally different genotypes, known as “supertypes.” Supertyping is used to partition haplotypes into groups based on amino acid properties that are assumed to affect antigen binding capability (Reche and Reinherz, 2003). Supertype clusters were predicted using Discriminant Analysis of Principal Components (DAPC) implemented with the adegenet package in R (vrs. 3.3.0; Jombart et al., 2010).

### Detection of Potential Parasites

Swab samples were assayed for *Bd* using duplicate quantitative polymerase chain reaction (qPCR; Boyle et al., 2004). To examine broader parasite diversity, DNA extracts were PCR-amplified with dual-indexed, barcoded broad eukaryote primers, and then sequenced on the Illumina MiSeq platform (Stoeck et al., 2010). Sequences were quality-filtered and processed using Quantitative Insights into Microbial Ecology (QIIME; Caporaso et al., 2010) and UCHIME2 (Edgar, 2016) and assigned taxonomy using Silva 97 and Genbank reference databases (**Supplementary Material**). Briefly, sequences that passed quality filtering were grouped into operational taxonomic unit (OTU) clusters at the standard microbial threshold of 97% similarity. Taxonomy was assigned to OTUs by comparing a representative sequence from each sequence cluster against the Silva 97 reference database using the BLAST search algorithm to find the closest match. Because this database has limited taxonomic resolution for certain groups (e.g., some Fungi contain few or no representative sequences in Silva 97), the representative sequence set was then compared against GenBank sequence database using the BLASTn search algorithm to confirm OTU identity.

OTUs were categorized as either potentially parasitic or non-parasitic using a standardized Google Scholar search. The search was performed with the parameters “[taxon name] AND parasite OR pathogen.” Resulting articles were examined manually to determine whether published accounts of the taxon as a parasite in either amphibians or other animal hosts existed.

Potential parasite OTUs (PP OTUs) were rarefied to 200 sequences (determined via visual examination of the minimum asymptotic point of read/sample histograms). Datasets with and without opportunistic pathogens/parasites were analyzed separately to provide liberal (i.e., all PP OTUs, including likely parasites and opportunistic PP OTUs) and conservative (i.e., only likely parasites, excluding opportunistic PP OTUs) estimates of potential parasite richness. The fraction of PP OTUs that were likely to be opportunistic OTUs (only opportunistic PP OTUs) were also analyzed to test whether loss of genetic diversity is associated with increased



opportunistic infections. For all three PP OTU fractions, alpha diversity metrics (Observed OTUs, Chao 1 index, and phylogenetic diversity) as well as beta diversity distance matrices were computed in QIIME and analyzed across groups in R.

Independent samples *t*-tests were used to examine associations between alpha diversity and site type (island vs. mainland) or MHC genotype (homozygous vs. heterozygous, supertype). Associations between beta diversity and genetic or geographic distance of host populations were tested with Mantel correlations to test the null hypothesis that parasite community structure is a function of geography rather than host properties. Simple Linear Regressions were used to determine the relationship between population-level MHC genetic diversity and  $\log_{10}$  mean parasite richness.

## RESULTS

### Neutral Genetic and MHC Diversity

Twenty-seven MHC IIB haplotypes that condensed into three functionally divergent superotypes were recovered from the *T. taophora* study populations (**Figure 1B**). At most two alleles were recovered from each individual frog. MHC IIB diversity was significantly lower in island populations in terms of nucleotide diversity [ $\pi$ , independent samples *t*-test,  $t_{(8)} = 3.209$ ,  $p < 0.012$ ; **Table 1**]. Island populations also showed trends toward lower MHC class IIB diversity in terms of allelic richness ( $N_A$ ; Mann-Whitney Test,  $U = 2$ ,  $p = 0.067$ ) and segregating sites *S* [*t*-test,

$t_{(8)} = 1.926$ ,  $p = 0.09$ ; **Table 1**]. MHC IIB allelic richness was positively associated with microsatellite allelic richness across all populations (Linear Regression weighted by sample size, one-tailed  $p = 0.04$ ,  $R^2 = 0.42$ ). There were no differences in observed or expected MHC IIB heterozygosity between island and mainland populations ( $H_O$  and  $H_E$ , independent samples *t*-tests,  $p > 0.05$  for each metric).

When all data were pooled and analyzed, there was a statistically significant signature of positive selection ( $dN/dS > 1$ ) at the MHC IIB locus ( $dN/dS = 2.811$ ,  $p = 0.003$ ). In addition, comparison of island vs. mainland populations also revealed signatures of positive selection for all groups (all coastal populations:  $dN/dS = 2.292$ ,  $p = 0.012$ ; all island populations:  $dN/dS = 2.843$ ,  $p = 0.003$ ). However, when populations were analyzed individually, 2/3 mainland populations showed signatures of positive selection while only 1/6 islands exhibited a  $dN/dS$  significantly  $> 1$  (**Table 1**). This is likely due to low allele number in island populations (only sites with four or more alleles had  $dN/dS$  ratios significantly  $> 1$ ).

Geographic distance showed no significant association with MHC IIB  $F_{ST}$  (partial Mantel test,  $-r = -0.0179$ ,  $p > 0.5$ ). To further examine whether neutral processes or selection were more likely influencing MHC class IIB diversity (see Radwan et al., 2009), we compared the MHC  $F_{ST}$  across all the *T. taophora* study populations with the overall  $F_{ST}$  averaged across all microsatellite loci sequenced by Duryea et al. (2015). The overall MHC  $F_{ST}$  value was 0.729, which falls above 2SE of the mean microsatellite  $F_{ST}$  ( $0.496 \pm 0.082$  SE).

**TABLE 1** | Summary statistics for microsatellite data (extracted from Duryea et al., 2015) and MHC IIB data.

Population	I/C	Microsatellite data				MHC IIB data									
		<i>n</i>	<i>N<sub>A</sub></i>	<i>H<sub>E</sub></i>	<i>H<sub>O</sub></i>	<i>n</i>	<i>N<sub>A</sub></i>	<i>H<sub>E</sub></i>	<i>H<sub>O</sub></i>	dN/dS	<i>p</i> (dN/dS)	<i>S</i>	<i>Pi</i>	Tajima's <i>D</i>	<i>p</i> ( <i>D</i> )
Couves Sul	I	17	4.1	0.60	0.55	30	2	0.47	0.43	1.266	0.104	2	0.0032	-0.6119	>0.10
Prumirim	I	26	3.8	0.46	0.51	22	4	0.17	0.09	<b>2.284</b>	<b>0.012</b>	10	0.0097	<b>-2.0756</b>	<b>&lt;0.05</b>
Porcos Pequena	I	37	3.5	0.46	0.43	20	1	0.00	0.00	0.957	0.17	1	0.0007	-1.1575	>0.10
Tamanduá	I	33	4.4	0.54	0.47	25	1	0.00	0.00	0	1	0	0.0000	-	-
As Ilhas	I	-	-	-	-	4	1	0.00	0.00	1	0.16	1	0.0043	0.5590	>0.10
Gatos	I	12	2.3	0.57	0.48	3	1	0.00	0.00	0	1	-	-	-	-
Couves Norte	I	17	4.1	0.60	0.55	7	2	0.50	0.43	-	-	-	-	-	-
Sununga	C	49	8.4	0.67	0.56	20	11	0.67	0.70	<b>2.022</b>	<b>0.023</b>	8	0.0204	0.1776	>0.10
Toque Toque	C	-	-	-	-	24	10	0.73	0.51	<b>1.854</b>	<b>0.033</b>	9	0.0141	-1.3337	>0.10
Barra do Una	C	19	8	0.71	0.59	20	2	0.1	0	0.445	0.329	3	0.0055	-0.8755	>0.10
All Coastal	C	-	-	-	-	-	-	-	-	<b>2.292</b>	<b>0.012</b>	-	-	-	-
All Island	I	-	-	-	-	-	-	-	-	<b>2.843</b>	<b>0.003</b>	-	-	-	-
All sites	I/C	-	-	-	-	-	-	-	-	<b>2.811</b>	<b>0.003</b>	14	0.0361	1.2923	>0.10

*I*, Sites are coded as island; *C*, coastal mainland; *N<sub>A</sub>*, including allelic richness; *H<sub>E</sub>*, expected heterozygosity; *H<sub>O</sub>*, observed heterozygosity; *S*, segregating sites; *pi*, nucleotide diversity. *dN/dS* and associated *p*-values for the MHC IIB locus was calculated in MEGA and *S*, *Pi*, and Tajima's *D* and associated *p*-values were calculated in DnaSP. Bold *dN/dS* values and Tajima's *D*-values indicate a significant departure from zero. Coastal populations had significantly higher MHC nucleotide diversity than island populations (*t*-test, *p* < 0.05), and also showed trends toward greater allelic richness (Mann-Whitney Test, *p* = 0.07) and more segregating sites (*t*-test, *p* = 0.09).

## Bd Infection Prevalence and Load Among Study Populations

All study populations exhibited low *Bd* loads (range 1–100 ZE with the majority ~10 ZE). Mean *Bd* infection load and prevalence did not significantly differ among populations (ANOVA, *p* = 0.2 for infection load among all *Bd* positive populations; *t*-test, *p* = 0.6 for prevalence between pooled mainland and pooled island populations; **Figure S1**). There was also no significant association between *Bd* load and neutral genetic diversity (*p* > 0.05 in Simple Linear Regression for both *H<sub>O</sub>* and *N<sub>A</sub>* of microsatellites).

## Potential Parasite Identification

After quality and chimera filtering, there were 845 total eukaryotic OTUs found across all samples. Eukaryotic OTUs that were determined through the standardized literature search to be potentially parasitic made up ~11% (97/845) of the total OTUs. The prevalence of coinfections was extremely high: 150/168 individuals with potential parasites hosted two or more potentially parasitic OTUs (hereafter PP OTUs), and a maximum of 18 PP OTUs was recovered from one individual (**Figure 2A**). PP OTUs were taxonomically diverse (**Figure 2B**), with 37 likely parasites and 60 likely opportunistic pathogens (**Table S1**).

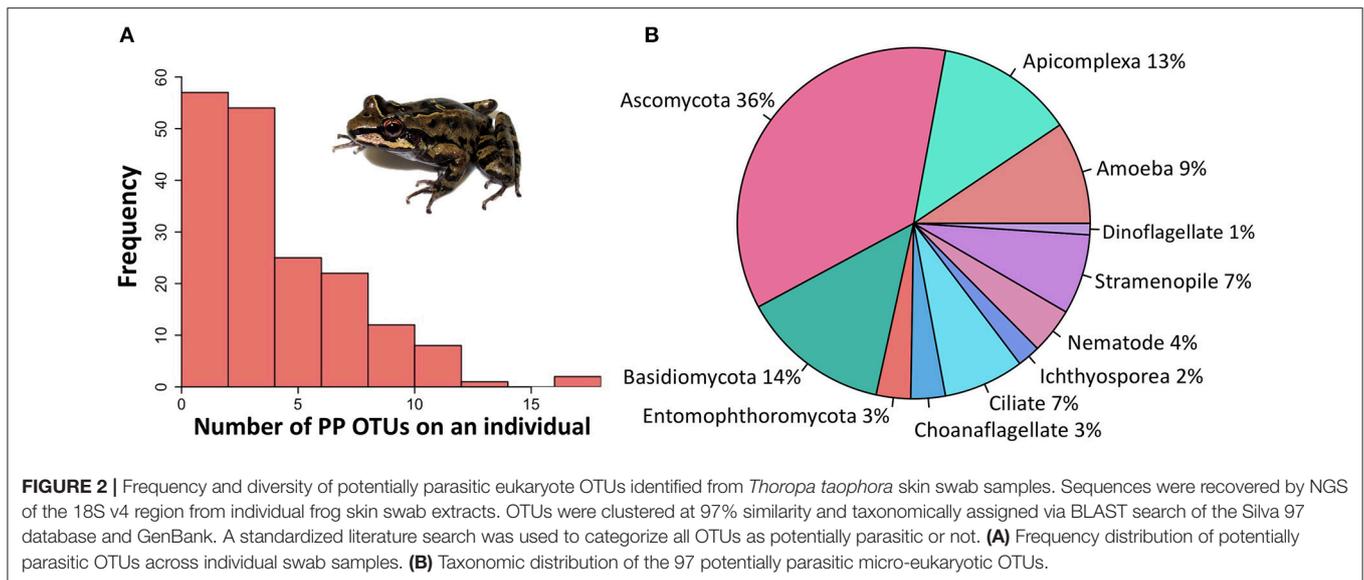
Many of the potential parasites were identified as endoparasites, such as Apicomplexa which are known to infect blood cells. Tissue and swab samples from eight individual frogs for which apicomplexan sequences were recovered in our 18S dataset were further analyzed to validate the amplification of internal parasites from skin swabs. In all eight of these samples, PCR-amplification with apicomplexan primers resulted in bands in sample pairs (tissue and swab samples from the same individual frog) for at least one of the two primer sets, with 4/8 sample pairs showing strong amplification with both primer

sets. Clean DNA sequences were obtained from sample pairs from 6/8 individual frogs. When the sequences were searched against GenBank using BLASTn, all six sample pairs matched the same apicomplexan identity: five of the tissue/swab sample pairs matched *Hepatozoon* sp., and the remaining pair matched *Adelina* sp.

## Potential Parasite Diversity and Distribution

Beta diversity of PP OTUs did not correlate with geographic or genetic distance (Mantel correlation, *p* > 0.05). Overall, alpha diversity of PP OTUs did not vary by site type (*t*-tests for observed OTUs, PD, and Chao1, *p* > 0.05). However, when opportunistic PP OTUs were excluded from analyses, PP OTU alpha diversity was lower in island populations (observed OTUs: 0.42 average PP OTUs in islands vs. 1.10 in mainland, Mann-Whitney test, *U* = 2,883.5, *p* < 0.01; PD: 0.25 island vs. 0.64 mainland, Mann-Whitney test, *U* = 2,841, *p* < 0.01; Chao 1: 0.42 island vs. 1.10 mainland, Mann-Whitney test, *U* = 2,883.5, *p* < 0.01). Site type did not contribute to differences in alpha diversity of opportunistic PP OTUs (*t*-tests for observed OTUs, PD, and Chao1, *p* > 0.05).

Richness varied by MHC IIB genotype for the overall PP OTU dataset and for the PP OTU dataset with opportunistics excluded, with fewer PP OTUs in MHC IIB homozygotes for both fractions (all PP OTUs: observed OTUs: 4.05 mean PP OTUs in homozygotes vs. 5.59 in heterozygotes, Mann-Whitney test, *U* = 3,889.5, *p* < 0.05; PD: 1.04 in homozygotes vs. 1.51 in heterozygotes, Mann-Whitney test, *U* = 3,697.5, *p* < 0.001; Chao 1: n.s., *t*-test, *p* > 0.05; PP OTUs with no opportunistics: observed OTUs: 0.45 mean PP OTUs in homozygotes vs. 1.3 in heterozygotes, Mann-Whitney test, *U* = 3,711.5, *p* < 0.001; PD: n.s., *t*-test, *p* > 0.05; Chao 1: 0.46 in homozygotes vs. 1.28 in



heterozygotes, Mann-Whitney test,  $U = 3,500.5$ ,  $p < 0.05$ ). When only opportunistic PP OTUs were analyzed, richness did not vary by MHC IIB genotype ( $t$ -tests,  $p > 0.05$  for all alpha diversity metrics).

MHC IIB Supertype had no effect on alpha diversity in the total PP OTU dataset. However, when opportunistics were excluded, PP OTU richness was significantly lower in frogs possessing Supertype 3 (observed OTUs: n.s.,  $t$ -test,  $p > 0.05$ ; PD: 0.18 in ST3+ vs. 0.48 in ST3-, Mann-Whitney test,  $U = 1485.5$ ,  $p < 0.05$ ; Chao1: 0.37 in ST3+ vs. 0.74 in ST3-, Mann-Whitney test,  $U = 1,486$ ,  $p < 0.05$ ). When only opportunistic PP OTUs were analyzed, frogs possessing MHC IIB Supertype 2 hosted a greater number of PP OTUs (observed OTUs: 5.04 in ST2+ vs. 3.33 in ST2-, Mann-Whitney test,  $U = 3,619.5$ ,  $p < 0.01$ ; PD: n.s.,  $t$ -test,  $p > 0.05$ ; Chao1: 5.26 in ST2+ vs. 3.36 in ST2-, Mann-Whitney test,  $U = 3,653$ ,  $p < 0.01$ ).

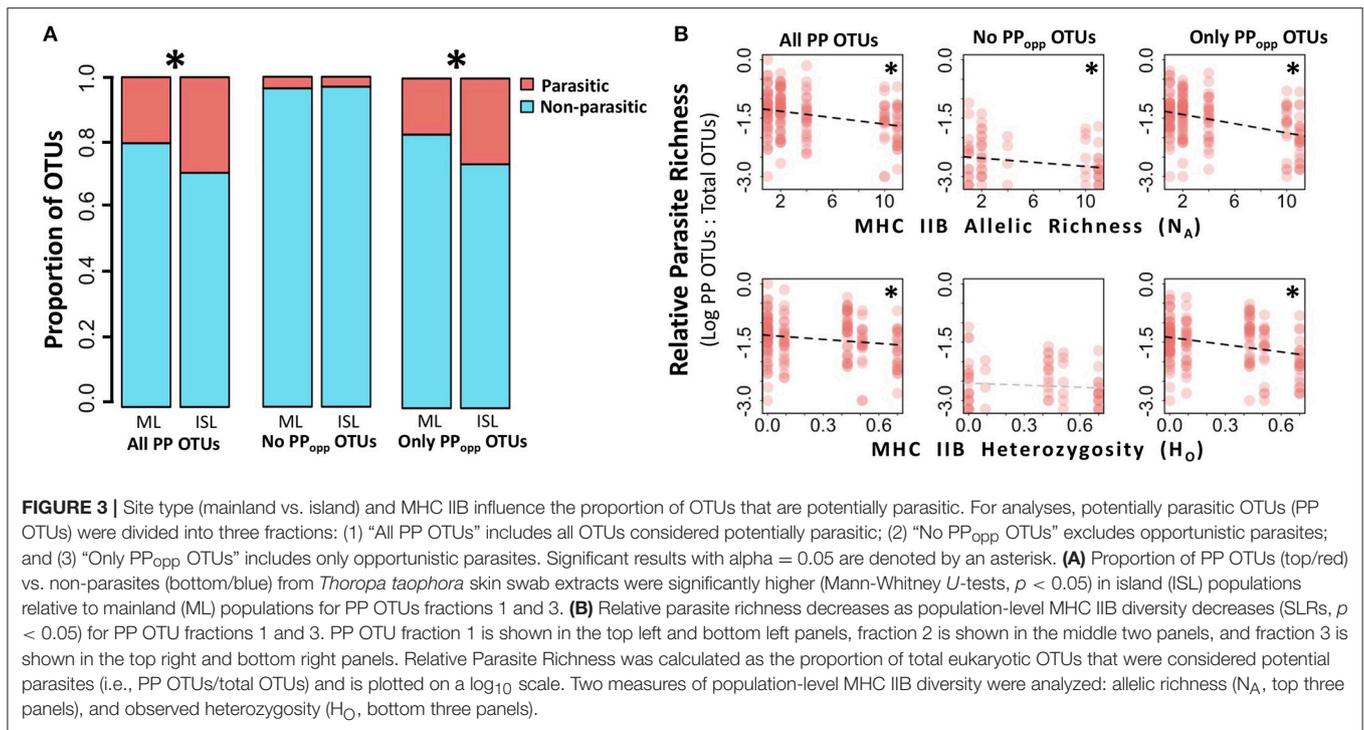
On average, fewer total eukaryotic OTUs were recovered from island frogs than mainland frogs (Mann-Whitney test,  $U = 2,787$ ,  $p < 0.01$ ). To evaluate relative parasite pressure among populations while accounting for differences in baseline eukaryotic diversity, the proportion of PP OTUs to total eukaryotic OTUs on each individual was calculated for further statistical analyses. Relative to mainland frogs, island frogs hosted proportionately more PP OTUs overall (0.29 mean proportion PP OTUs:total OTUs in islands vs. 0.20 in mainland, Mann-Whitney test,  $U = 4,963$ ,  $p < 0.001$ ) and proportionately more opportunistic PP OTUs (0.26 mean opportunistic PP OTUs:total OTUs in islands vs. 0.17 in mainland, Mann-Whitney test,  $U = 5,072$ ,  $p < 0.001$ ; **Figure 3A**), but similar levels of only non-opportunistic PP OTUs ( $t$ -test,  $p > 0.05$ ). On the population level, MHC class IIB diversity was negatively associated with a greater proportion of PP OTUs for all three fractions of PP OTUs (all PP OTUs: SLR,  $\beta = -0.025$ ,  $p < 0.001$ ,  $R^2 = 0.16$  for  $N_A$ ,  $\beta = -0.247$ ,  $p < 0.001$ ,  $R^2 = 0.07$  for  $H_O$ ; only non-opportunistic PP OTUs: SLR,  $\beta = -0.015$ ,  $p < 0.05$ ,  $R^2 = 0.08$  for  $N_A$ ,  $\beta = -0.145$  but n.s. with  $p > 0.05$  for  $H_O$ ; only opportunistic

PP OTUs:  $\beta = -0.031$ ,  $p < 0.001$ ,  $R^2 = 0.21$  for  $N_A$ ,  $\beta = -0.338$ ,  $p < 0.001$ ,  $R^2 = 0.12$  for  $H_O$ ; **Figure 3B**).

To distinguish between effects of site type and MHC IIB diversity on proportional diversity of PP OTUs, these analyses were repeated for only mainland populations. The negative association between proportion of PP OTUs remained present and significant for all PP OTUs (SLR,  $\beta = -0.02$ ,  $p < 0.05$ ,  $R^2 = 0.10$  for  $N_A$ ,  $\beta = -0.27$ ,  $p < 0.01$ ,  $R^2 = 0.11$  for  $H_O$ ) and for only opportunistic PP OTUs (SLR,  $\beta = -0.026$ ,  $p < 0.01$ ,  $R^2 = 0.15$  for  $N_A$ ;  $\beta = -0.357$ ,  $p < 0.01$ ,  $R^2 = 0.16$  for  $H_O$ ). While only non-opportunistic PP OTUs showed negative associations (negative regression slopes) with the two metrics of MHC IIB population-level diversity, these relationships were no longer significant when only mainland populations were analyzed (SLR,  $p > 0.05$ ).

## DISCUSSION

The ecological and evolutionary impacts of long-term fragmentation on land-bridge island fauna has been studied in a number of systems (Karr, 1982; Aguilar et al., 2004; Hurston et al., 2009; Estrada-Villegas et al., 2010; Duryea et al., 2015; Belasen et al., 2016; Santonastaso et al., 2017). In a previous study examining immunogenetic diversity in island vertebrates, MHC diversity remained high while overall diversity dropped (Aguilar et al., 2004). This led us to predict that island *T. taophora* populations would display a similar pattern. Contrary to our expectations, we found that MHC class IIB diversity is reduced in island populations relative to their mainland counterparts (**Table 1**) and appears to have eroded at a rate similar to the loss of neutral diversity. This suggests a relatively strong effect of genetic drift on the MHC IIB locus. However, the larger overall dN/dS, the high level of MHC genetic differentiation ( $F_{ST}$ ) relative to neutral loci, as well as a lack of MHC geographic structure support the action of positive selection. These patterns



may have been generated by divergent selection, and suggest that the divergence observed in MHC IIB is not solely a product of genetic isolation but rather that selection may be leading to acceleration of differences among populations.

Although the idea that high MHC diversity is common remains prevalent in the literature, MHC diversity has been shown to correlate with overall diversity in a number of species, suggesting that demographic processes that affect neutral loci can extend to the MHC (reviewed in Radwan et al., 2009). Low MHC diversity is not necessarily an indication of imminent extinction; very low MHC diversity has been demonstrated in stable and viable wildlife populations (Ellegren et al., 1993) and entire species (Slade, 1992). Nevertheless, reduced diversity at immunogenetic loci could reduce resistance to infections: hosts would be less likely to possess matched resistance alleles to novel or invading pathogens, while pathogens could adapt more quickly or escape immune cell recognition in less immunogenetically variable hosts (Radwan et al., 2009; Siddle et al., 2010).

To examine whether fragmentation and genetics influence infections in *T. taophora*, we first conducted a standard assay for the fungal amphibian pathogen, *Bd*. The presence of *Bd* in almost all study populations but at very low loads (Figure S1) suggests *Bd* resistance in this species, enzootic dynamics of *Bd* in the study area, and/or unsuitable environmental conditions. *T. taophora* inhabits lowland coastal areas and can tolerate some contact with seawater. The salinity of the habitat (Stockwell et al., 2015) as well as high and variable temperatures may negatively impact the growth of *Bd*. Experimental studies are needed to distinguish between *Bd*

resistance in *T. taophora* and low environmental suitability for *Bd*.

To gain a clearer picture of infections in *T. taophora*, we screened for a diversity of micro-eukaryotes in skin swab samples. Our Next-Generation Sequencing (NGS) analysis combined with a standardized literature review recovered 97 potentially parasitic (PP) micro-eukaryote OTUs, and up to 18 unique PP OTUs found on a single individual (Figure 2A). These results demonstrate a high frequency and diversity of coinfections, and underscore the limitations of using targeted assays for only one or few known pathogens. Although there were no differences among populations in *Bd* prevalence or load, there were significant patterns when we examined the PP OTUs identified with NGS. As we hypothesized, island populations exhibited higher parasitism, i.e., proportionately more PP OTUs than mainland populations (Figure 3A and Table 2), despite higher PP OTU diversity at mainland sites. This was only true for the total PP OTU dataset and the dataset only including likely opportunistic PP OTUs, but not for the more conservative PP OTU dataset that excluded opportunistics. This implies that opportunistic PP OTUs are driving this trend, although this result could also be due to a lack of statistical power in the very small conservative PP OTU dataset ( $n = 37$  OTUs). A higher diversity of PP OTUs on islands could be attributed to higher contact between hosts and/or hosts and parasites in the limited island geographic area. However, island frogs hosted significantly fewer total eukaryotic OTUs than mainland frogs, implying that the microbiome of island frogs may be depauperate despite potentially higher contact rates.

Our results also indicate significant associations between PP OTUs and MHC class IIB. On the individual level, PP

**TABLE 2** | Summary of predicted outcomes and observed study results.

Variable	Prediction	Prediction supported?		
		All PP OTUs	Likely PP OTUs	Opportunistic PP OTUs
Genetic Diversity	Greater parasitism in genetically eroded populations: island frogs should host more PP OTUs	Yes: Island frogs hosted proportionately more overall PP OTUs than mainland frogs	No: Island populations had lower PP OTU alpha diversity; proportion of PP OTUs:Total OTUs was not different between islands and mainland	Yes: Island frogs hosted proportionately more opportunistic PP OTUs than mainland frogs
	Negative association between genetic diversity and parasitism: higher genetic diversity = lower parasite diversity	Yes: Population-level MHC IIB diversity ( $H_O$ and $N_A$ ) was negatively associated with proportion of total OTUs that were PP OTUs	Yes: Population-level MHC IIB diversity ( $N_A$ ) was negatively associated with proportion of total OTUs that were PP OTUs	Yes: Population-level MHC IIB diversity ( $H_O$ and $N_A$ ) was negatively associated with proportion of total OTUs that were opportunistic PP OTUs
MHC Genotype	Heterozygote advantage: heterozygotes should host fewer PP OTUs	No: Heterozygotes hosted a higher diversity of PP OTUs	No: Heterozygotes hosted a higher diversity of PP OTUs	No: There were no differences in opportunistic OTU diversity between heterozygotes and homozygotes
	MHC IIB Supertype affects parasite diversity	No: No effects of MHC Supertype on PP OTU diversity	Yes: Supertype 3 was associated with fewer PP OTUs	Yes: Supertype 2 was associated with more opportunistic PP OTUs

OTU alpha diversity was higher in MHC IIB heterozygotes both when the entire PP OTU dataset was analyzed and when opportunistic PP OTUs were excluded (Table 2). It remains unclear whether this relationship is driven by balancing selection on the MHC in the presence of a higher diversity of pathogens and parasites, or whether heterozygotes exhibit higher tolerance for pathogens and parasites, resulting in higher apparent infections. MHC class IIB supertype was also associated with differences in PP OTU richness. Supertype 3 was associated with fewer non-opportunistic PP OTUs, whereas Supertype 2 was associated with greater numbers of opportunistic PP OTUs than those possessing either Supertype 1 or Supertype 3. These results suggest that MHC IIB genotype can influence infection dynamics, and specifically that in *T. thoropa* Supertype 3 may be associated with the greatest resistance against likely parasites. More research is needed to determine the relationship between parasite richness and disease outcomes, as well as the evolutionary consequences of elevated parasite diversity on host immunogenes.

On the population level, reduced MHC class IIB diversity corresponded with proportionally more PP OTUs (PP OTUs: total eukaryotic OTUs; Figure 3B and Table 2). To address the possibility that these relationships were confounded by genetic structure or other factors covarying with site type (such as environmental differences between sites), we excluded island populations and re-analyzed the data, and recovered the same negative associations between MHC IIB diversity and proportional richness PP OTUs. The largest negative slopes and highest  $R^2$ -values were observed for the dataset only including opportunistic PP OTUs (across all populations:

$\beta = -0.031$ ,  $R^2 = 0.21$  for  $N_A$ ,  $\beta = -0.338$ ,  $R^2 = 0.12$  for  $H_O$ ). This relatively strong negative relationship would be expected if population-level MHC IIB diversity reflects the immunocompetence of individuals, as immunocompromised individuals tend to be more susceptible to opportunistic infections (Anaissie, 1992).

The negative associations of all three PP OTU fractions with MHC IIB diversity suggest that populations that lose allelic diversity at the MHC class IIB locus may be subject to increased parasitism. Epidemics select for rare alleles in the surviving population, a mechanism known as negative frequency-dependent selection (Lively and Dybdahl, 2000). Loss of rare alleles through the loss of immunogenetic diversity may thus increase population-level susceptibility to new pathogen invaders (Bernguber et al., 2013). This dovetails with the finding that rare immunogenetic types have been associated with increased disease resistance in natural populations (Schwensow et al., 2007). Due to limited sample sizes, we did not possess the statistical power to determine whether rare alleles (haplotypes) were associated with lower parasite richness in *T. taophora*. Nonetheless, the association of population-level MHC IIB diversity with lower PP OTU richness suggests that populations with more rare alleles may experience fewer infections.

Our approach for identifying a broad diversity of parasites does not come without caveats. First, the specificity of the taxonomic match reported for each OTU is dependent upon whether data from closely related taxa occur in the reference database. Nearly half (39/97) of our potentially parasitic OTUs had a closest match from GenBank of <97% (Table S1),

which is the commonly accepted (and arguably conservative) sequence similarity cutoff below which microbial organisms are considered distinct species. For example, one OTU in our dataset (“New.CleanUp.ReferenceOTU912”) matched *Perkinsus qugwadi*, a species which is known to parasitize mollusks (Blackbourn et al., 1998). However, this represents a match of low identity (83%), indicating that our OTU is likely a related organism. Lower on the list of closest GenBank matches for our OTU is an unidentified alveolate parasite of the frog *Rana sphenoccephala* (Davis et al., 2007). This further supports that our OTU represents an unidentified but likely parasitic eukaryote.

A second caveat of our approach is that we categorized some OTUs as potentially parasitic based on known parasitism in vertebrate taxa other than amphibians. We argue that it is reasonable to treat these as potential parasites because (1) many of these OTUs are present on a number of individuals from a number of populations (Table S1), which reduces the likelihood that these are incidental or transient; and (2) they represent poorly-studied taxa, particularly in non-human animals. A number of the PP OTUs that are non-specifically listed as “animal parasites” are classified within the Apicomplexa, which is an entirely parasitic phylum.

Third, the presence of these PP OTUs in a sample does not necessarily imply disease: many parasitic microbes can be commensal in healthy individuals or at low loads. However, the presence of parasite organisms is necessary for disease, implies a less than healthy microbiome (Willing et al., 2010), and is likely to activate host immune responses even in the absence of symptomatic disease. While interpretations of our dataset are limited by a lack of demonstrated pathology, we present a preliminary study that advances understanding of the diversity and distribution of potentially parasitic microbes inhabiting the skin of non-human animal hosts. Complementary approaches including microscopy and/or experimental infections would be useful in future studies to confirm parasitism by PP OTUs identified with a similar NGS-based parasite screening approach.

We note that we are unable to exclude other factors that may contribute to infection susceptibility, including contributions by other genetic loci (including other immunogenetic loci), effects of individual-level genome-wide diversity or heterozygosity (Acevedo-Whitehouse and Cunningham, 2006), aspects of innate immunity (Richmond et al., 2009), or environmental influences on immunity and/or disease. In addition, because the MHC region we have amplified is relatively short (~120 bp), our analysis may underestimate the number of haplotypes among populations. However, despite some level of ascertainment bias, we have recovered a genetic pattern that is significantly associated with variation in potential parasite diversity.

Lastly, we note that the remaining ~89% of eukaryotes that were amplified from the skin samples could be ecologically important as well. Eukaryotes in the amphibian skin microbiome have only recently come under study (Kueneman et al., 2016), though bacteria found in the amphibian skin microbiome are known to play significant roles in amphibian host health. In particular, some bacteria are known to inhibit *Bd*

(Myers et al., 2012) and may contribute to disease resistance (Harris et al., 2009). Preliminary research on eukaryotes suggests that fungi isolated from frog skin may in reality contribute more to *Bd* resistance than bacteria (Kearns et al., 2017). Symbiotic micro-eukaryotes have the potential to serve as competitors or hyperparasites of harmful microbes in the host-associated microbiome. Our method of combining NGS with a survey of available literature can be applied in future research to tie host-associated microbiota data with demonstrated ecological functions of the microbiome.

The results of our study have several implications. First, the effects of fragmentation on immunogenetics can be strong, although these may be dependent on the focal taxon and the conditions under study. Second, using targeted assays to study specific pathogens may underestimate infections, to the extent that analyses may be completely blind to significant ecological patterns. Third, fragmentation may increase disease susceptibility by altering host genetics. Our results support prioritizing genetic diversity in vulnerable wildlife populations to improve survival and resilience. Future studies of field infections should bear in mind that diverse coinfections in amphibians may be highly prevalent, and thus should consider a broad diversity of pathogens and parasites if infection status is a variable of interest.

## DATA AVAILABILITY STATEMENT

The supporting data and sequences for this study can be found in the GenBank (MK348627 - MK348653 and MK350361 - MK351204).

## ETHICS STATEMENT

All methods were approved by the Institutional Animal Care and Use Committee of the University of Michigan (protocols PRO00005605 and PRO00007691).

## AUTHOR CONTRIBUTIONS

AB, TJ, and LT conceived of the study. AB, TJ, LT, and DL coordinated the field and lab work. AB, TJ, and LT collected field samples. AB performed the labwork. AB and MB performed the data analysis. All authors provided contributions to the writing and gave final approval before submission.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2018.00236/full#supplementary-material>

- pathogen *Batrachochytrium dendrobatidis*. *Conserv. Biol.* 26, 135–141. doi: 10.1111/j.1523-1739.2011.01801.x
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