The Impacts of Habitat Fragmentation on Amphibian Genetics and Health in the Brazilian Atlantic Forest Biodiversity Hotspot

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Ecology and Evolutionary Biology)
in the University of Michigan
2019

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Dedication

In memory of my grandmother, Daisy Belasen,
who taught me to be resilient and to make my own happiness.
Throughout my PhD I tried to remember her words:
כל עכבה לטובה.
(Every delay is for good reason.)

Acknowledgements

I would not have been able to complete this dissertation without the support of collaborators, scientific colleagues, friends, and family. I am grateful to my PhD committee members Johannes Foufopoulos, Meg Duffy, and Lacey Knowles, who each contributed their expertise, knowledge, and resources to my dissertation, and helped me grow as a scientist and as a person. Thank you to Ivette Perfecto and John Vandermeer, for making me feel welcome at your lab meetings and NWAEG meetings, and for inspiring me to develop a more impactful research plan. I also would not have survived graduate school without the help, support, patience and kindness of a number of EEB administrators and staff, including Gail Kuhnlein, Jane Sullivan, Cindy Carl, and Kati Ellis. Thank you all for your support throughout this process.

I would like to acknowledge my collaborators, especially those from Brazil: Felipe Toledo, who made all of my fieldwork possible, provided me with endless support in Brazil, and connected me with his own network of Brazilian herpetologists; Gui Becker, who helped me develop ideas and allowed me to run with his pet study system in São Luiz; Domingos da Silva Leite, my fellow showtune enthusiast, who made me feel at home in his lab at UNICAMP; and Mirco Solé, who (despite the fact that I was a complete stranger) welcomed me and provided me with the resources to complete my fieldwork in Bahia. I would like to acknowledge the many Brazilian students and field technicians who helped me organize my field trips, collect and analyze samples, improve my Portuguese, and learn to enjoy life in the moda brasileira, including Carol Lambertini, Paula Morão, Tsunami Moreno, Tamilie Carvalho, Carlão Almeida, Camila Torres (Colombiana pseuda-Brasileira), Simone Dena, Meg Nogueira, Joice Ruggeri, Guilherme Alves, Leandro Tacioli, Vini Hansser, Rafael "Hobbit" Benetti, Renato Martins, Cesar Alexandre, Amanda Piffer, Lucas Forti, Felipe Andrade, Isa Haga, João Roberto, and many others.

I would like to acknowledge the inspirational scientist friends I made during my time in graduate school, who helped me recover from failures and celebrate successes. I would particularly like to thank my dear friends Liana May, Erin Burkett, Nayiri Haroutunian, Dan Katz, John Guittar, Kevin Li, and Kinsey Brock, who I met during my time in the School of Natural Resources and Environment and continued to support me during my PhD; and Clarisse Betancourt-Roman, Sujal Phadke, Joanna Larson, Clara Shaw, Jill Myers, Kevin Amses, Rob Powers, Marian Schmidt, Chatura Vaidya, Kristel Sanchez, Andrea Belgrade, and Talia Moore, who I met during my time as a PhD student. You brightened my dark Michigan winter days, and helped me become a better scientist and human being. I am grateful to my support network of #HERpers (women herpetologists), and particularly Whitney Gentry Walkowski, Helen Plylar, Priya Nanjappa, and Kirsten Hecht – *behind every successful woman is a group text.*

I would also like to acknowledge the support of my family: my siblings, the four other A's: Ari, Amy, Amanda, and Abby, all of whom are pursuing their academic, professional, and personal dreams in their own ways. And I cannot express enough gratitude to my parents, Alan and Susan Belasen, who showered me with love and support when I needed it the most, never declined a request to proofread a proposal or paper, and always encouraged me to keep chasing my dreams. Thank you for reminding me where I came from and what I value throughout the arduous process of completing my PhD.

I am endlessly grateful to my partner, Erik Helwig, who has patiently and enthusiastically supported me since Day 1 of graduate school. Thank you for traveling with me, cooking for me, proofreading for me, helping me study for prelims, making me laugh, buying me chocolate, and being there to support me whether things were going wrong or right.

Finally, I would like to acknowledge the James Lab, my family away from home. In particular I would like to thank Rebecca Clemons, my first mentee, the #1 qPCR star, who I have proudly watched grow into a wonderful scientist, and who has become a collaborator and friend. Finally, to my advisor Tim James: you have been an infinitely patient and supportive mentor and collaborator, and taught me by example how to do good science while making sure to have a good time. Thank you for taking a chance on this herpetóloga louca.

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Abstract

Amphibians are declining worldwide due to emerging infectious disease and habitat modification. Although these stressors overlap in time and space, we know little about their interactions. For example, habitat fragmentation reduces genetic diversity in wildlife, and genetic diversity is correlated with disease resistance according to theoretical and laboratory work. However, little is known about the relationship between genetic diversity and disease incidence in wild populations. In my dissertation, I evaluated the impacts of habitat fragmentation on potential disease susceptibility in amphibians of the Brazilian Atlantic Forest (BAF), one of the most biodiverse but heavily fragmented areas on the planet.

In Chapters 2-3, I sampled populations of a widespread coastal frog species (Cycloramphidae: *Thoropa taophora*) across a set of land-bridge islands that represent 12,000-20,000 year old habitat fragments, which I compared with mainland "control" populations. In Chapter 2, I examined the impacts of overall genetic diversity loss due to long-term isolation on islands on (1) immunogenetic (MHC IIB) diversity and (2) susceptibility to microeukaryote infections in a single host frog species. Contrary to previous studies that found high immunogenetic diversity in genetically impoverished populations, I found that inbred island populations exhibited significantly lower MHC IIB diversity than mainland populations. My results also showed that island populations and MHC IIB homozygotes were subject to more infections by diverse potentially parasitic microbes. In Chapter 3 I examined the relationship between immunogenetics and the assembly and diversity of the host-associated microbiome. I found that microbiome diversity was dependent on MHC IIB genotype, with heterozygotes

hosting a higher diversity of potentially beneficial microbes. My results also strongly imply that there are interactions between bacteria and eukaryote microbes in the microbiome which have been overlooked by previous studies that focus only on the bacterial amphibian skin microbiome.

In Chapters 4-5, I compared the impacts of two different types of habitat modification in the mainland BAF: (1) ~200 year old forest fragments set in a "sea" of intensive cattle pasture, and (2) shaded cacao plantations that serve as less aversive anthropogenic habitats. I compared frog populations found in both habitat types with "control" populations in continuous preserved forests. I sampled six frog host species to examine how the impacts of habitat modification vary according to species ecology: half of the frogs were high-dispersing habitat generalists and half were low-dispersing habitat specialists. In Chapter 4, I evaluated genetic diversity and isolation across these habitats and species. I found that while only generalist species showed reduced genetic diversity in forest fragments embedded in intensive agriculture, only specialists showed genetic isolation. Populations in rustic agricultural areas exhibited similar genetic diversity as those in preserved forests and relatively low genetic isolation, implying that rustic agriculture is less aversive to sensitive animals. In Chapter 5, I examined immunogenetic diversity and infections in a subset of these populations. I found that across all species, fragmented populations exhibited reduced immunogenetic diversity and increased infections. Immunogenotype influenced infections by both the pathogenic fungus *Batrachochytrium dendrobatidis* and by apicomplexan blood parasites.

Taken together, my results suggest that the impacts of habitat modification on amphibian health are significant, and can include loss of overall genetic diversity, loss of immunogenetic diversity, loss of microbiome diversity, and increased infections. This relationship may help explain the recent rise of infectious diseases in amphibians and other wildlife species worldwide.

Chapter 1: Introduction

Amphibians, bats, bees, and snakes are currently in decline due to recently emerged infectious diseases. Perhaps the most puzzling are the disease-associated amphibian declines: although we have a good understanding of the geographic distribution and effects of major amphibian diseases, disease outcomes remain relatively unpredictable. In particular, a number of questions about amphibian disease remain unanswered, including: (1) Why are some amphibian species able to persist during epidemics while others decline or go extinct? (2) Why are some individual amphibians *within a single species* able to survive epidemics while others die? In other words, why is there variation in disease susceptibility among and within host species? I hypothesize that the answers lie in the interactions between disease susceptibility and other stressors to which amphibians are highly susceptible. In particular, habitat modification is a globally widespread stressor of amphibians that can reduce community diversity, genetic diversity, and immunocompetence, all of which may increase disease. **In my dissertation research, I test the hypothesis that changes in host genetics associated with habitat modification increase potential disease susceptibility in amphibians.** Understanding the interactions between habitat modification and disease is critical to disease mitigation and conservation efforts across the globe.

The amphibian pathogen *Batrachochytrium dendrobatidis* (*Bd*), the causal agent of the disease chytridiomycosis, has been implicated in the global declines of at least 500 amphibian species and the extinction of 90 amphibian species over the last four decades (Carvalho et al.

2017; Lips et al. 2006; Scheele et al. 2019). *Bd* occurs on every continent except Antarctica, with the most infamous *Bd*-associated declines reported from Australia, Central America, and the western US (Daszak et al. 1999). The loss of amphibians due to *Bd* has had large-scale and long-lasting impacts on natural ecosystems (Whiles et al. 2006), making amphibian management and *Bd* mitigation top conservation priorities.

However, to the detriment of conservation efforts, the basis for *Bd* susceptibility or resistance is still poorly understood. While many populations have gone extinct following *Bd* outbreaks, some rebound within a few years or months, while others appear to experience little to no declines (Briggs et al. 2010; Savage et al. 2011). The mechanisms behind this variation in susceptibility remain to be pinned down, but mounting evidence points to both environmental (James et al. 2015; Scheele et al. 2019) and genetic factors (Savage and Zamudio 2011; Bataille et al. 2015; Scheele et al. 2019). Moreover, *Bd* is not the only pathogen of concern in amphibians. With their unprotected and physiologically active skin exposed to moist, microbe-rich habitats, amphibians are subject to infection by a number of pathogens, including Ranaviruses, pathogenic fungi, and protozoans (Gleason et al. 2014; Martel et al. 2013; Speare et al. 1994; Desser et al. 1995). Much attention has been paid to *Bd* because of the worldwide scale and severity of *Bd*-associated declines, and the majority of amphibian disease studies focus on only *Bd* or only a single other pathogen or parasite species. However, coinfections can produce more severe epidemics than single infections (Susi et al. 2015), and genetic and immune mechanisms of disease susceptibility can be general (Bevan 1987; Richmond et al. 2009). Therefore, only examining a single pathogen or parasite offers a limited view of the overall health and susceptibility of amphibians. **I designed my dissertation research to evaluate how**

differences in host genetic diversity due to landscape modification impact variation in susceptibility to infection by a diversity of parasitic species.

How might host genetic diversity impact disease susceptibility? Decades of research have shown that genetic diversity on both the population-level and individual-level improves resistance to stressors including disease (Reed and Frankham 2003; Sgrò et al. 2011; Clark et al. 2011; Pearman and Garner 2005). Genes involved in the immune response, such as immunogenes in the vertebrate-wide Major Histocompatibility Complex (MHC) gene family, play central roles in the disease response. The MHC gene family occurs in all vertebrates and is composed of two classes that present antigen peptides. Class I MHC genes are involved in the response to viral pathogens, while Class II MHC genes are involved in the response to extracellular pathogens such as bacteria and fungi (Bevan 1987). In amphibians, the MHC IIB locus is associated with interspecific *Bd* susceptibility (Bataille et al. 2015). MHC IIB has also been linked with intraspecific variation in population-level and individual-level disease outcomes in correlational studies of natural populations (Kosch et al. 2016), and experimental infection trials (Savage and Zamudio 2011; Kosch et al. 2018).

This relationship between MHC genotype and disease susceptibility is due to the functional role of MHC genes in specific pathogen responses. MHC genes code for MHC molecules that bind to antigen peptides and signal immune cells to mount the immune response (Richmond et al. 2009). MHC genotype determines the shape of the peptide binding region (PBR) and thus the repertoire of pathogens that can be bound (recognized) to stimulate an immune response (Piertney and Oliver 2006). For these reasons, MHC diversity can be maintained at very high levels due to balancing or diversifying selection to maximize organismal disease resistance and fitness (Doherty and Zinkernagel 1975; McClelland et al. 2003;

Whitehorn et al. 2011). At the population level, negative frequency-dependent selection can occur whereby rare MHC alleles provide an advantage as pathogens evolve to overcome common resistance alleles (Takahata and Nei 1990). In previous studies of the amphibian MHC IIB and *Bd,* there has been evidence for both heterozygote advantage and for specific alleles conferring resistance (Savage and Zamudio 2011, 2016). Because landscape modification can cause changes in the local parasite and pathogen community (Lafferty 2012) and also alter the selective environment (Hernández-Gómez et al. 2019), we may expect immunogenetic allele frequencies to change with landscape modification. Results from a recent study suggest that agricultural land use is associated with adaptive selection on amphibian immunogenes (Hernández-Gómez et al. 2019). Still, much remains to be understood about the relationships between habitat fragmentation and immunogenetic diversity across amphibian taxa that vary in ecology and disease susceptibility.

Aside from direct functional roles in the immune response, host genetics may also indirectly influence disease susceptibility through influencing disease-relevant phenotypes, such as the diversity of the host-associated microbiome. In amphibians, the skin microbiome serves as the first line of defense against skin pathogens such as *Bd* (Harris et al. 2009; Muletz et al. 2012). According to previous studies, amphibian skin microbiomes represent non-random subsets of the microbes present in the local environment, and vary more among than within amphibian host species (Kueneman et al. 2014; Walke et al. 2014), implying that the microbiome is adaptive and species-specific. Although much remains to be understood regarding the relationship between amphibian host genetics and microbiome assembly within species, a study on a laboratory model frog species (*Xenopus laevis*) suggested that frogs with different immunogenotypes have different microbiome composition (Barribeau et al. 2012). More research is available on the role

of genetics in human gut microbiomes, and there is evidence that genetic factors (including immunogenes) and genetic diversity have impacts on microbiome diversity and assembly (Benson et al. 2010; Blekhman et al. 2011; Marietta et al. 2015; Zoetendal et al. 2001; Goodrich et al. 2016). These studies suggest that host genetics can influence the microbiome, which may constitute another mechanism by which host genetics influences disease susceptibility.

Unfortunately, the genetic diversity of wildlife is being threatened worldwide by landscape modification including anthropogenic habitat fragmentation, the process by which natural continuous habitats are divided and reduced into isolated patches. Depending on the sensitivity of the wildlife species surviving in fragmented landscapes, fragmentation may result in complete isolation and consequent inbreeding within the remaining habitat patches. Combined with the increased effects of random genetic drift in these small isolated populations, genetic diversity can quickly erode (Newmark 1995; Zuidema et al. 2017). The majority of this fragmentation occurs in tropical areas (Perfecto and Vandermeer 2010) which are also hotspots of biodiversity, making habitat fragmentation one of the biggest threats to global species conservation.

However, there are many factors that can impact the relationship between fragmentation and genetic diversity, including time since fragmentation, the quality of the matrix between habitat patches, and the ecology and sensitivity of wildlife species. First, a minimum amount of time is required to observe the impacts of fragmentation. Similar to the "Extinction Debt," whereby extant species will be lost in the future due to current fragmentation (Tilman et al. 1994), the impacts of genetic drift and inbreeding are also subject to time lags and only become apparent after a minimum number of generations (Mona et al. 2014). Therefore, the negative impacts of fragmentation may be difficult to detect in very recently fragmented systems. Second,

matrix quality can mediate the impacts of landscape modification. For example, in landscapes where rustic farming practices are employed, wildlife species dispersal and survival seem to be little impacted relative to more intensively modified landscapes (Perfecto and Vandermeer 2010). Third, species ecology can influence the impacts of habitat fragmentation. Species with generalist ecologies may experience fewer negative impacts of fragmentation (Harrison and Bruna 2012), although species with high dispersal may be more impacted by fragmentation than low-dispersing species that essentially already experience local isolation (Funk et al. 2005). Therefore, it is important to consider a range of time, landscape, and species contexts to understand the impacts of habitat fragmentation in complex and biodiverse tropical systems.

To evaluate the impacts of habitat fragmentation on genetic diversity and disease susceptibility across a range of contexts, I sampled frogs in the Brazilian Atlantic Forest, one of the most biodiverse and heavily fragmented tropical regions in the world. I collected samples over three field seasons in three areas that exemplify different challenges to frog genetic diversity: (1) a system of small islands (São Paulo), inhabited by highly inbred frog populations that have been isolated from mainland populations for 12,000-20,000 years; (2) ~200 year old habitat fragments (São Paulo) alongside a preserved forested area; and (3) ~200 year old habitat fragments (Bahia) alongside preserved areas that contain patches of native forest and patches of rustic agroforest. In each region, I sampled frog populations found in fragmented areas (island or forest fragment) as well as populations found in continuous areas (mainland or continuous forest that has been preserved or modified). This parallel design allowed me to test the impacts of fragmentation on host amphibian populations across a range of time, matrix type, and species ecology contexts.

My first two data chapters (Chapters 2-3) focus on the island-mainland system and address the effects of long-term island isolation on genetic diversity and potential disease susceptibility. I sampled populations of the cycloramphid frog *Thoropa taophora* across seven islands and four mainland sites in a small region of the Brazilian Atlantic Forest (SE São Paulo). Focusing on a single species allowed me to control a number of factors (*e.g.*, differences in species ecology and/or evolutionary history) so that I could examine the effect of genetic diversity on infection susceptibility. I collected frog tissue samples to examine genetics, and skin swab samples to investigate parasites and other microbes on the frogs.

In Chapter 2, I analyzed a genetic region involved in disease susceptibility, the Major Histocompatibility Complex (MHC) IIB locus. Based on previous findings that MHC diversity can be extremely high even in inbred island populations (Aguilar et al. 2004), I hypothesized that island populations would exhibit high MHC diversity despite long-term inbreeding. I also tested the hypothesis that long-term habitat fragmentation increases infections by characterizing parasites found in skin swab samples using DNA amplification and sequencing. Surprisingly, MHC IIB diversity declined in the island populations of *T. taophora.* Although none of my populations harbored significant *Bd* infections, I found a broad diversity of eukaryotic pathogens present in my study populations. I found that infection rates by these pathogens were higher in inbred island populations and associated with MHC IIB genotype over all populations, such that MHC IIB heterozygotes hosted fewer infections. I hypothesized that this effect could be mediated through impacts of MHC IIB genotype on other microbes that interact with pathogens on amphibian skin.

To address this hypothesis, in Chapter 3 I examined the host-associated microbiome as a mechanism by which host genetics might alter potential disease susceptibility. The island-

mainland system provided a set of populations that differ in genetic background, both in terms of overall diversity and MHC IIB diversity, allowing me to evaluate how these factors impact microbiome diversity and assembly. Previous skin microbiome studies in amphibians have largely focused on bacteria, but this likely provides an incomplete picture of the diverse microbial community found on amphibian skin. Therefore, I simultaneously sequenced bacterial and eukaryotic microbial DNA from frog skin swabs to examine the full range of effects that fragmentation might have on the microbiome community. I found that microbiome structure was correlated with geographic distance as well as fragmentation, and microbiome diversity was lower in MHC IIB homozygotes. These results support the hypothesis that MHC genotype influences infections by impacting the overall microbiome community, and provide a novel link between immunogenetics and health in amphibians.

My first two data chapters examined the long-term impacts of habitat fragmentation on frog health. However, the island-mainland system is limited in that it does not reflect the current impacts of recent habitat fragmentation. In addition, very few host species occur across the coastal region, with only *T. taophora* found on many of the islands in the region. With a higher diversity of potential hosts, host species ecology and variable effects of fragmentation across species could significantly influence disease outcomes. Therefore, for my third and fourth data chapters (Chapters 4-5), I sampled recently fragmented landscapes in the mainland Brazilian Atlantic Forest (southeastern São Paulo and southern Bahia). The majority of the landscape in these regions consists of tiny patches of forest (the majority 0-50 ha in area) in a sea of intensive agriculture (cattle pasture), which prevents dispersal among forest patches by sensitive animals such as amphibians. To determine the effects of this fragmentation on amphibian genetic diversity and infections, I sampled frogs in forest fragments and nearby continuous preserved

forests in both regions. To evaluate how matrix quality alters the impacts of fragmentation on amphibians and their parasites, I also sampled frogs from continuous modified habitats in southern Bahia known as cabruca, which is a type of agroforestry in which cacao trees are grown under forest canopy. Specifically, I sampled frogs in cabruca areas that varied from rustic (cacao planted beneath natural forest trees) to managed (cacao planted beneath planted rubber tree canopy). With these samples, I was able to test the effects of a range of fragmentation types across a range of frog species.

I collected genetic and skin swab samples from six frog species that vary from exceedingly hardy (habitat generalists) to highly sensitive to habitat fragmentation (forest specialists). I sampled frogs from a range of ecologies to gain a more complete picture of the effects of fragmentation on tropical amphibian communities, as these represent a diversity of reproductive modes and dispersal abilities. Understanding the range of fragmentation effects across species is also important for predicting disease outcomes for generalist pathogens like *Bd*, because the effects of fragmentation on host species interactions (including disease transmission) will change with different species communities and fragmentation contexts.

In Chapter 4, I tested the effects of fragmentation across the range of modified habitat types (fragments in intensive cattle pasture vs. rustic cabruca vs. managed cabruca) on dispersal and genetic diversity across six focal frog species. Half of these species were habitat generalists and half were forest specialists. I hypothesized that all species would exhibit genetic diversity loss due to fragmentation, but that this loss would be more severe in forest specialists. I also hypothesized that forest specialists would show the highest level of genetic isolation in fragments. I tested these hypotheses using a reduced representation library DNA sequencing approach (double digest restriction site-associated DNA sequencing, ddRAD). Although my

hypotheses were somewhat supported, I found a somewhat paradoxical pattern. While specialists on the whole did appear to exhibit genetic isolation due to fragmentation while generalists did not, generalists showed genetic erosion with fragmentation but specialists exhibited *increased* genetic diversity in fragments. My review of the literature suggests that this latter result may be due to deeper historical population structure in forest specialists compared with habitat generalists (Carnaval 2006; Rodríguez et al. 2015).

In Chapter 5, I built on this genetic data and examined the effects of fragmentation on parasitism by looking at (1) MHC IIB diversity and (2) infection rates across fragmented and continuous populations of the six study species in fragmented and continuous preserved habitats in São Paulo and Bahia. I quantified *Bd* infections in both sampling areas, but because lowland Bahia is not an ideal environment for *Bd*, I also quantified malaria-like parasite infections (Apicomplexans) in both areas. Similar to my island study results, I found erosion in MHC IIB diversity with fragmentation. As I hypothesized, apicomplexan infections were higher in Bahia than in São Paulo. Both pathogens were associated with MHC IIB diversity and MHC IIB genotypes, but showed opposite patterns: as MHC IIB diversity increased, *Bd* prevalence decreased but Apicomplexan loads increased. Combined with the result that two MHC IIB genotypes (Supertype 4 and Supertype 5) were associated with higher *Bd* prevalence but lower Apicomplexan loads, my results suggest a potential tradeoff in resistance to these two pathogens mediated by MHC IIB. Compared with the results from the island-mainland system, the results of my final two chapters are more directly applicable to contemporary populations responding to recent habitat fragmentation (<500 years before present). These provide important data on the more nuanced effects of different types of landuse change on wildlife populations, and may

provide support for adopting ecologically-sound agricultural practices in tropical centers of biodiversity.

My dissertation research has integrated fields of study that are usually separate – genetics, geography, epidemiology, and microbiology. By combining techniques and theoretical concepts from disparate fields, I have performed innovative research of natural populations to test hypotheses that have been primarily supported only through theoretical modeling or laboratory experiments. Taken together, my four data chapters address the interplay of habitat modification, host genetics, and disease susceptibility. The results are applicable to the management and conservation of a diversity of wildlife taxa that are vulnerable to habitat fragmentation and disease.

Chapter 2: Long-Term Habitat Fragmentation is Associated with Reduced MHC IIB Diversity and Increased Infections in Amphibian Hosts[1]

Abstract

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.

[1] Published as: Belasen, A. M., Bletz, M.C., da Silva Leite, D., Toledo, L. F., James, T. Y. 2019. Long-term fragmentation is associated with reduced immunogenetic diversity and increased infections in amphibian hosts. Frontiers in Ecology and Evolution, 6, 1-2. doi: 10.3389/fevo.2018.00236

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.; Ellison et al. 2012; Ilmonen et al. 2008; Spielman et al. 2004) and in studies of natural populations (Ellison et al. 2011; Pearman and Garner 2005; Roca et al. 2010). 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; Hambuch and Lacey 2002; Landry et al. 2001), 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 (Deter et al. 2008; Schwensow et al. 2007).

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 (Adrian V. S. 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., 2018).

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 (Cushman 2006; Kiesecker et al. 2001). 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 (Arens et al. 2007; Lesbarrères et al. 2005). 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; Gervasi et al. 2015; Bielby 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* (Bataille et al. 2015; Savage and Zamudio 2011; 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 (Gleason et al. 2014; Martel et al. 2013; Speare et al. 1994). 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 (Yildirimhan et al. 2006; Dyer 1991). 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 (Arenas et al., 2014; Mona et al., 2014; Tilman et al., 1994). 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 (R. C. 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 (Fig. 2-1a). 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 (R. C. 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 (Bokermann 1974; Sazima 1971; 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 4,000-28,000 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 = 4-30 per site, 179 total) were individually sampled from each of the 10 study populations between January-March 2015 (IACUC protocols for humane animal care and use PRO00005605 and PRO00007691; 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 ~120bp fragment of the MHC IIB locus was PCR-amplified from tissue DNA extracts (May and Beebee, 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 (π) were calculated in DnaSP (Version 5; Librado and Rozas, 2009). T-tests were used to evaluate differences in S and π 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 supertypes were recovered from the *T. taophora* study populations (Fig. 2-1b). At most two alleles were recovered from each individual frog. MHC IIB diversity was significantly lower in island populations in terms of nucleotide diversity (π, independent samples t-test, $t_8 = 3.209$, $p < 0.012$; Table 2-1). 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 2-1). 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 greater than 1 (Table 2-1). This is likely due to low allele number in island populations (only sites with four or more alleles had dN/dS ratios significantly greater than 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 ± 0.082 SE).

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; Fig. 2-4S). There was also no significant association between *Bd* load and neutral genetic diversity (p > 0.05 in Simple Linear Regression for both H_O and N_A 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 (Fig. 2-2a). PP OTUs were taxonomically diverse (Fig. 2-2b), with 37 likely parasites and 60 likely opportunistic pathogens (Appendix 2-1).

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 proportionally more opportunistic PP OTUs (0.26 mean opportunistic PP OTUs:total OTUs in islands vs. 0.17 in mainland, Mann-Whitney test, $U = 5,072$, $p < 0.001$; Fig. 2-3a), 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; Fig. 2-3b).

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; Estrada-Villegas et al. 2010; Belasen et al. 2016; Duryea et al. 2015; Hurston et al. 2009; 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 1-1) 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 (Fig. 2-4S) 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 (Fig. 2-2a). 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 (Fig. 2-3a; Table 2-2), 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 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 2-2). 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; Fig. 3b; Table 2-2). 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 (Berngruber 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. Approximately 40% (39/97) of our potentially parasitic OTUs had a closest match from GenBank of $\leq 97\%$ (Table 1-

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 sphenocephala* (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 1-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, Woodhams, Treuren, 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.

Acknowledgments

We thank Paula Morão and Luis Moreno for assistance in field logistics, field work, and labwork; Vinicius Hansser, Amanda Piffer, and Cesar Alexandre for assistance in the field; Carlos Almeida for providing site coordinates; Rebecca Clemons and Nicholas Farrugia for assistance with labwork; Alisha Quandt, William Davis, Thibaut Jombart, and Anna Savage for assistance with bioinformatics and data analysis; and Kelly Zamudio and Celio Haddad for assistance with fieldwork planning. We also thank two anonymous reviewers for providing feedback on earlier versions of the manuscript. We wish to acknowledge funding from NSF (OISE-1159513), CNPq (300980/2014-0), and a Block Grant from the U. Michigan Dept. of Ecology and Evolutionary Biology.

Data Availability Statement

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

Tables

Table 2-1: Summary statistics for microsatellite data and MHC IIB data.
Microsatellite data were extracted from Duryea et al. (2015). Sites are coded as island (I) or coastal mainland (C), including allelic richness (NA), expected heterozygosity (HE), observed heterozygosity (HO), segregating sites (S), and nucleotide diversity (pi). 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. $p = 0.09$).

		Microsatellite data				MHC IIB data									
Population	**I/C**	**n**	**N_A**	**H_E**	**H_O**	**n**	**N_A**	**H_E**	**H_O**	**dN/dS**	**p (dN/dS)**	**S**	**Pi**	**Tajima's D**	**p (D)**
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	**2.284**	**0.012**	10	0.0097	**-2.0756**	**< 0.05**
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
Tamandua	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	**2.022**	**0.023**	8	0.0204	0.1776	> 0.10
Toque Toque	C	-	-	-	-	24	10	0.73	0.51	**1.854**	**0.033**	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	-	-	-	-	-	-	-	-	**2.292**	**0.012**	-	-	-	-
All Island	I	-	-	-	-	-	-	-	-	**2.843**	**0.003**	-	-	-	-
All sites	I/C	-	-	-	-	-	-	-	-	**2.811**	**0.003**	14	0.0361	1.2923	> 0.10

Table 2-2: 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

Figures

Figure 2-1: Locations of Thoropa taophora frog populations and MHC IIB Haplotype network.
Sample sizes of adult frogs captured and sampled are listed in Table I-1. **(a)** Map of populations sampled in January-February 2015. Inset map shows the location of the study area in southeastern Brazil. Mainland sites are represented with triangles and islands with circles. Colors of markers correspond to colors of populations in haplotype network. **(b)** Haplotype network showing the MHC IIB locus amplified from *Thoropa taophora*. Black dots on branches indicate the number of mutations between haplotypes. Circle size is proportional to haplotype frequency. Haplotypes are annotated with corresponding supertype designations (ST1, ST2, or ST3).

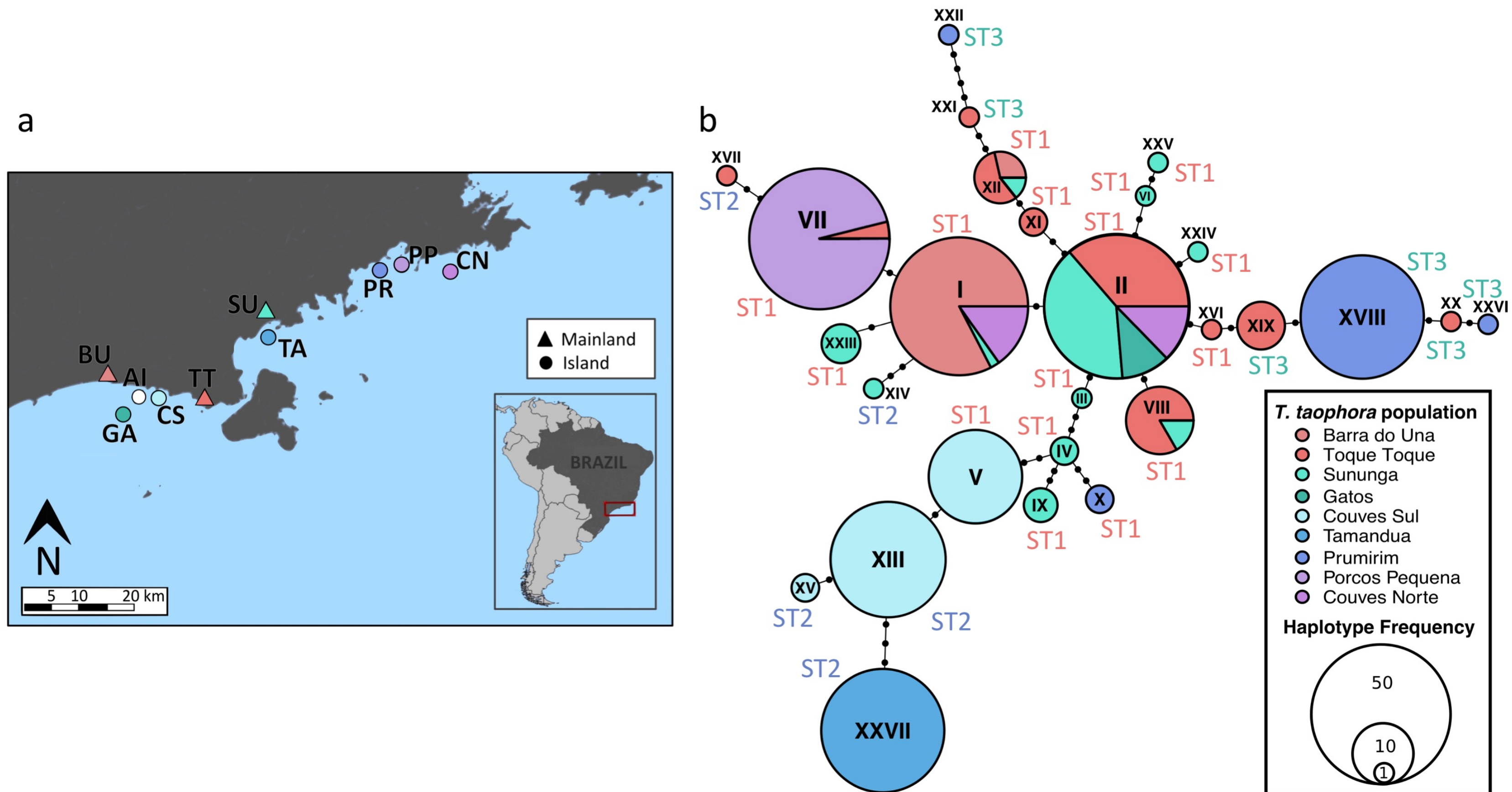


Figure 2-2: Frequency and diversity of potentially parasitic eukaryote OTUs identified from *Thoropa taophora* skin swab samples.
Sequences were recovered by NGS of the 18S v4 region from individual frog skin swab extracts. OTUs were clustered at 97% similarity and taxonomically assigned via BLAST search of the Silva 97 database and GenBank. A standardized literature search was used to categorize all OTUs as potentially parasitic or not. (a) Frequency distribution of potentially parasitic OTUs across individual swab samples. (b) Taxonomic distribution of the 97 potentially parasitic micro-eukaryotic OTUs.

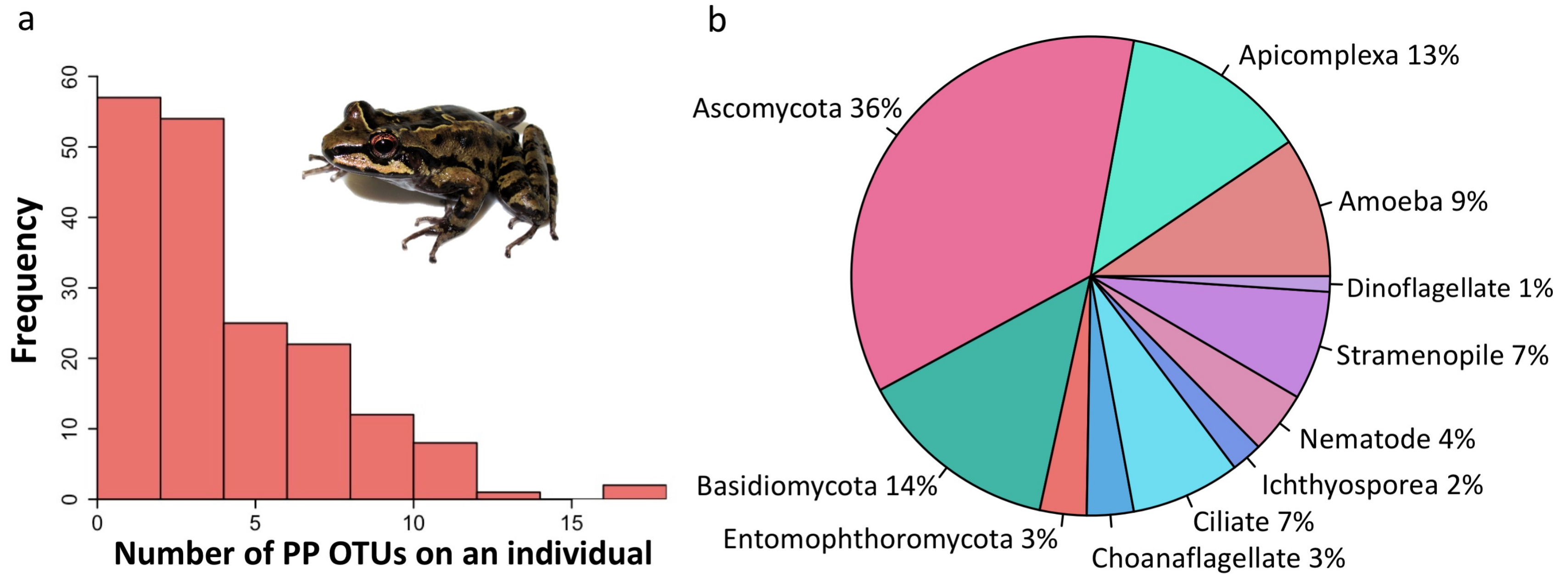
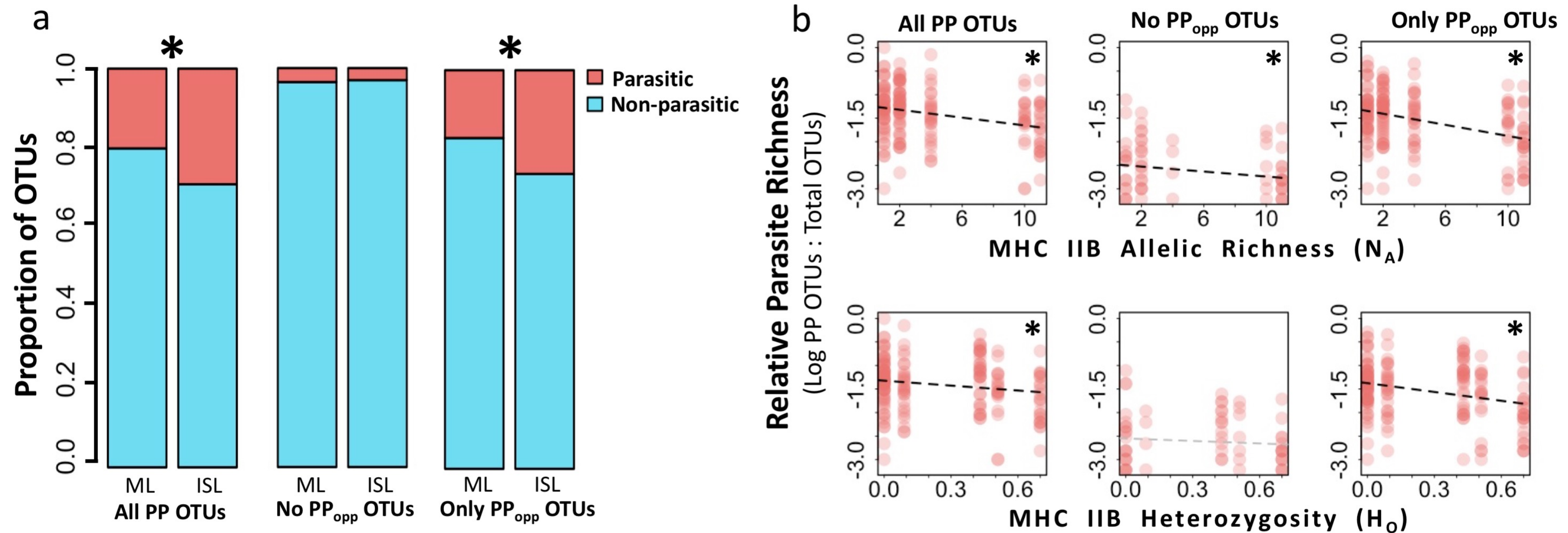


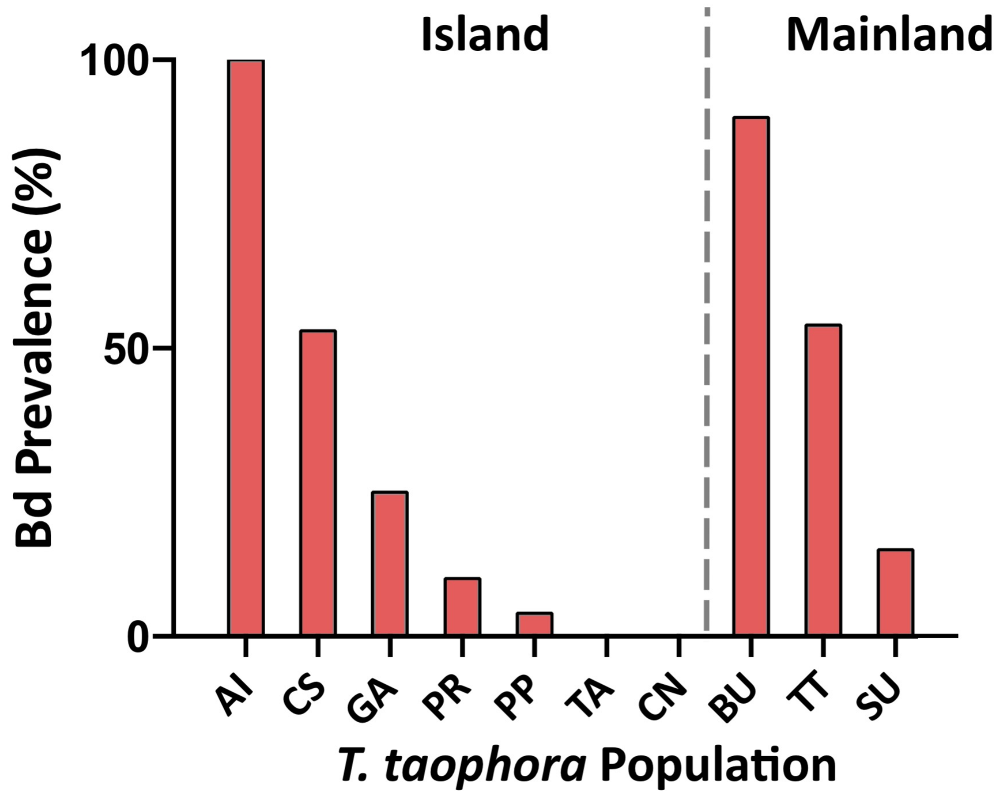
Figure 2-3: Site type (mainland vs. island) and MHC IIB influence the proportion of OTUs that are potentially parasitic.
For analyses, potentially parasitic OTUs (PP OTUs) were divided into three fractions: (1) "All PP OTUs" includes all OTUs considered potentially parasitic; (2) "No PP_{opp} OTUs" excludes opportunistic parasites; and (3) "Only PP_{opp} OTUs" includes only opportunistic parasites. Significant results with alpha = 0.05 are denoted by an asterisk. **(a)** Proportion of PP OTUs (top/red) vs. non-parasites (bottom/blue) from *Thoropa taophora* skin swab extracts were significantly higher (Mann-Whitney U tests, $p < 0.05$) in island (ISL) populations relative to mainland (ML) populations for PP OTUs fractions 1 and 3. **(b)** Relative parasite richness decreases as population-level MHC IIB diversity decreases (SLRs, $p < 0.05$) for PP OTU fractions 1 and 3. PP OTU fraction 1 is shown in the top left and bottom left panels, fraction 2 is shown in the middle two panels, and fraction 3 is shown in the top right and bottom right panels. Relative Parasite Richness was calculated as the proportion of total eukaryotic OTUs that were considered potential parasites (i.e., PP OTUs/total OTUs) and is plotted on a log_{10} scale. Two measures of population-level MHC IIB diversity were analyzed: allelic richness (N_A, top three panels), and observed heterozygosity (H_O, bottom three panels).



SUPPLEMENTAL INFORMATION AND FIGURES

Figure 2-4S: Prevalence of Batrachochytrium dendrobatidis across island and mainland populations of Thoropa taophora.

Prevalence is expressed as a percentage of the total individuals that tested positive for Bd ($\geq$ 1 zoospore equivalent) in a standard qPCR assay. There were no differences in prevalence between sites (ANOVA, p > 0.05) or site types (island vs. mainland, t-test, p > 0.05).



Supplemental Molecular and Analytical Methods

Immunogenetic analyses

MHC Class II genes were PCR-amplified from *T. taophora* toeclip DNA extracts using degenerate amphibian MHC primers BCF6 and BobomSR (May and Beebee 2009) and Sanger sequenced (Applied Biosystems 3730xl DNA Analyzer) at the University of Michigan Sequencing Core. Chromatograms were visually examined to check quality and identify single nucleotide polymorphisms (SNPs) in Sequencher (Version 5.3). NCBI BLASTp search of the MHC IIB region was used to verify the correct amino acid reading frame. Haplotypes were predicted using Phase (Version 2.1.1) (Stephens et al. 2001) to obtain allele frequencies, which were used to calculate allelic diversity (N_A) and observed and expected heterozygosity (H_o and H_e). Segregating sites (S) and nucleotide diversity (π) were calculated in DnaSP (Version 5; Librado & Rozas, 2009). T-tests were used to evaluate differences in S and π among island and coastal populations using t-tests, and simple linear regression was used to statistically compare MHC N_A and heterozygosity with microsatellite N_A and heterozygosity extracted from Duryea et al. (Duryea et al. 2015). All statistical analyses and verification of assumptions of parametric statistics were conducted in R (Version 3.3.0; Lighten, Van Oosterhout, & Bentzen, 2014).

MHC IIB sequences were translated, aligned, and placed into a maximum-likelihood tree with MHC IIB sequences from a diversity of amphibians (Bataille et al. 2015) in MEGA (vrs. 6.06-mac; Tamura et al. 2013). The ratio of non-synonymous to synonymous nucleotide changes (dN/dS) ratios and associated p-values for departure from the neutral expectation (dN/dS = 1, no selection) were calculated for each population and for island vs. mainland populations in MEGA. Nucleotide sequences were used to calculate Tajima's D and the p-value associated with its

departure from a neutral expectation (D = 0, random evolutionary processes) (Tajima 1989) for each population in DnaSP. To examine evolutionary and geographic patterns across immunogenetic alleles in the study populations, an MHC haplotype network was produced and visualized and the fixation index (F_{ST}) was calculated using the pegas package in R (Paradis 2010). We also calculated F_{ST} using data from the same populations to obtain mean and standard error estimates of genetic differentiation across all microsatellite alleles (data extracted from Duryea et al., 2015).

MHC haplotypes were condensed into functionally different genotypes, known as "supertypes." Supertyping is used to partition haplotypes into groups based on amino acid (AA) properties that are assumed to be affect antigen binding capability. The haplotype alignment was tested for positively selected sites (PSS) using PAML (vrs. 4.8; Yang, 2004). Nine codon positions that constituted PSS were extracted from the alignment and translated into a concatenated AA sequence. A matrix of physicochemical AA properties (z1-z5) was then constructed for each haplotype at each of the nine codon positions. This matrix was used to predict supertype clusters 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 first assayed for *Bd* using duplicate quantitative polymerase chain reaction (qPCR) (Boyle et al. 2004). qPCR standards (Brazilian *Bd*-GPL strain CLFT 023) containing zoospore genomic equivalents (ZE) of 1-1,000,000 zoospores were concurrently run in triplicate on each reaction plate to quantify experimental sample *Bd* loads. If only one sample

replicate amplified, the sample was run a second time. Samples were considered positive if they contained ≥1 ZE in at least two replicates.

To examine broader parasite diversity, DNA extracts were PCR-amplified with dual-indexed, barcoded broad eukaryote primers TAReuk454FWD1 and TAReukREV3 (for the 18S v4 region), and then pooled and sequenced on the Illumina MiSeq platform (Stoeck et al. 2010). The resulting sequences were quality-filtered and processed using Quantitative Insights into Microbial Ecology (QIIME; Caporaso et al., 2010). Sequences were clustered into operational taxonomic units (OTUs) using a 97% similarity threshold. Taxonomy was assigned using the BLAST search algorithm against the Silva 97 reference database. Chimeras were identified and filtered out using UCHIME2 (Edgar 2016). Sequences assigned to the host frog species or other non-target non-microbial species (*i.e.*, Streptophyta and Vertebrata) were removed from the dataset. Because a large proportion of OTUs were identified only to high taxonomic levels using the Silva database, all OTUs were compared against GenBank using BLASTn to confirm identity or identify a more specific taxonomic match.

The potential parasite OTUs recovered included endoparasites. Molecular analysis of skin swabs has been previously used to detect endoparasites (viruses) in amphibians (Gray et al. 2012). To further validate this approach for the detection of eukaryotic endoparasites, a subset of both swab and tissue samples from the same individual frogs were PCR-amplified using primers that amplify bloodborne apicomplexan parasites (Hep300/Hep900 (Ujvari et al. 2004) and HEMO1/HEMO2 (Perkins and Keller 2001)). PCR products obtained from swabs and tissues from the same individual frogs were Sanger sequenced to determine whether the same endoparasites detected in tissues were also detected in swabs.

Chapter 3: Geography, Host Genetics, and Microbial Interactions Structure the Skin Microbiome of Fragmented Brazilian Atlantic Forest Frog Populations[2]

Abstract

The skin microbiome plays a significant role in host health, but the importance of factors including host genotype, immunity, and microbial interactions in microbiome diversity and assembly remain unclear. We sampled naturally fragmented (island and mainland) frog populations to examine (1) the effects of geography and host genetic diversity on skin microbiome diversity and structure; (2) the structure of microbial eukaryotic and bacterial co-occurrence networks; and (3) the associations of bacteria known to affect growth of the fungal amphibian pathogen *Batrachochytrium dendrobatidis* (*Bd*) with other skin microeukaryotes. We performed an amplicon-based study using 180 *Thoropa taophora* skin swabs collected from seven island and three mainland populations in the southeastern Brazilian Atlantic Forest. Microbiome structure was correlated with geographic distance, and microbiome diversity was lower in genetically eroded island populations. Microbiome diversity was also associated with genotype at an expressed immunity locus (MHC IIB). Our network analysis revealed higher connectivity when both eukaryotes and bacteria were included, implying ecological interactions occur among Domains. Lastly, bacteria previously shown to affect *Bd* growth did not show broad antifungal properties, as there were not clear patterns with microbiome fungi. Our findings emphasize the

[2] This chapter is in preparation for publication and includes coauthors Maria Riolo, Mariana Lyra, L. Felipe Toledo, and Timothy Y. James.

importance of considering both bacterial and microeukaryotic components of the microbiome, and suggest that caution should be applied in utilizing probiotic strategies for disease management in wild amphibians.

Introduction

The host-associated microbiome has recently captured the attention of wildlife disease researchers seeking to understand and predict disease-associated wildlife population declines. Research on the skin microbiome is burgeoning in the field of amphibian disease, in which a majority of studies focus on the disease chytridiomycosis caused by the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). *Bd* and other pathogens have been linked to severe amphibian declines around the world since at least the 1970s (Lips et al. 2006; Olson et al. 2013; Carvalho et al. 2017). However, in the Eastern United States, plethodontid salamander populations showed no evidence of disease-associated declines despite the presence of *Bd* in the environment (C. Muletz et al. 2014). In a series of foundational studies (many of which were performed *in vitro*), bacteria cultured from salamander skin were correlated with reduced disease risk (Harris et al. 2006, 2009; C. R. Muletz et al. 2012). Further studies pointed to antifungal metabolite production by certain species of bacteria as the main mechanism of reduced disease (Harris et al. 2009; Myers et al. 2012; Woodhams et al. 2014). These findings among others gave rise to the concept of characterizing and potentially manipulating amphibian microbiome bacteria as a means to determine *Bd* susceptibility and mitigate disease-associated amphibian declines (Bletz et al. 2013; Woodhams et al. 2014; Piovia-Scott et al. 2017; Voyles et al. 2018).

Despite extensive research on specific *Bd*-inhibitory bacteria, much remains to be understood about the diversity and assembly of the overall amphibian skin microbiome,

including the roles of non-bacterial taxa (but see Kueneman et al. 2016; Kearns et al. 2017) and interactions between bacteria and non-bacterial microbes other than *Bd*. A diversity of microeukaryotes including fungi, microscopic metazoans, and protists have been previously identified on amphibian skin using high-throughput sequencing (Kueneman et al. 2017; Belasen et al. 2019). In studies examining microbial eukaryotes, fungi comprised the dominant eukaryotic taxon on adult amphibians (Kueneman, Woodhams, Van Treuren, et al. 2016), and explained more variation in *Bd* susceptibility than bacteria (Kearns et al. 2017). From studies in other host-microbe systems, symbiotic fungi are known to serve important roles in protecting host organisms against fungal pathogens (*e.g.*, mycorrhizae and endophytes in plants; Gao et al. 2010; Newsham et al. 1995). Fungi also serve as hyperparasites, *i.e.*, parasites of pathogens/parasites. For example, the cryptomycete fungus *Rozella allomycis* parasitizes chytrid fungi in the genus *Allomyces* (Gleason et al. 2012). Less is known about the symbiotic roles of protists, although microeukaryotes have been shown to impact health (Hoffmann et al. 2014; Holler et al. 2014) and immune function (Graham 2008) in mammals. Thus, eukaryotic members of the microbiome could be equally important as bacteria in determining disease susceptibility in vertebrates such as amphibians. Without an understanding of the interactions between microbiome bacteria and microeukaryotes, it is impossible to predict the potential microbiome-wide effects of proposed measures to manipulate bacteria to control *Bd* outbreaks.

In addition, few studies to date have examined the mechanisms that determine animal microbiome assembly and diversity. From research on humans, it is known that microbiome assembly and diversity can depend on many factors, including: (1) geography (Yatsunenko et al. 2012), although this may be confounded with both environmental and social context; (2) genetic factors and genetic diversity (Benson et al. 2010); and (3) immunogenetics, *i.e.*, genetic factors

related to immune function (Blekhman et al. 2011; Marietta et al. 2015), hypothetically due to interactions between immune cells and microbes including commensals and pathogens. Amphibian skin microbiome communities have been shown to be non-randomly selected from the environment, related to host species identity, and variable with host genotype (Kueneman et al. 2014; Walke et al. 2014), but it remains unclear whether geography or host factors determine the assembly of the amphibian skin microbiome. It also remains to be determined whether intraspecific genetic variation in amphibians impacts microbiome diversity and assembly. Prior studies on the model frog *Xenopus laevis* suggest that immunogenes in the major histocompatibility complex (MHC) are associated with variation in microbiome composition (Barribeau et al. 2012). Genetic control on microbial assembly suggests that related individuals are more likely to harbor similar microbiomes. Indeed, associations between genetic and microbiome similarity have been shown in humans through comparisons of monozygotic versus dizygotic twins (Zoetendal et al. 2001; Goodrich et al. 2016). However, the specific genes involved in assembly of the microbiome remain to be identified in humans or amphibians.

In a number of amphibian species, genetic diversity has been compromised due to anthropogenic habitat fragmentation (Allentoft and O'Brien 2010). Habitat fragmentation results in small, isolated host populations that undergo genetic erosion over time (Newmark 1995; Zuidema et al. 2017). Genetic erosion in fragmented populations has been observed at neutral loci as well as immunogenetic regions (Belasen et al. 2019) which may have important impacts on microbiome structure (Blekhman et al. 2011). In addition, fragmentation may cause a decline in microbial transmission, which in turn may alter microbial interactions and networks in host-associated microbiomes. The effects of habitat fragmentation are subject to time lags (Tilman et al. 1994) whereby genetic erosion resulting from inbreeding may not be detectable for several

generations following a fragmentation event; thus the effects of fragmentation are difficult to detect in recently fragmented populations. Historically fragmented populations offer an opportunity to examine the effects of genetic erosion on the microbiome and broader animal health.

We evaluated the effects of long-term habitat fragmentation on the amphibian skin microbiome using a historically fragmented model system in the Brazilian Atlantic Forest. This system consists of dozens of land-bridge islands, which were connected to the mainland prior to the Pleistocene but were naturally separated from the mainland 12,000-20,000 years ago via sea level rise (Suguio et al. 2005) and thus represent ancient forest fragments. Contemporary insular frog populations were once part of contiguous coastal populations, and are now isolated to the islands (R. C. Bell et al. 2012; Duryea et al. 2015). Using this geographic setting, we examined the impacts of both geography and host genetics on skin microbiome diversity and structure. We used amplicon-based high-throughput DNA sequencing to analyze skin swab samples collected from a single frog species (*Thoropa taophora* [Cycloramphidae]) found across both coastal mainland and island sites. The island populations of *T. taophora* have experienced fragmentation-induced genetic erosion at both neutral and immunogenetic loci (Duryea et al. 2015; Belasen et al. 2019). We also utilized a database of amphibian microbiome bacterial isolates to test for ecological relationships between *T. taophora* skin bacteria with known effects on *Bd* growth and other microeukaryotes found in the *T. taophora* microbiome including fungi and protists. Our study was designed to address the following research questions: (1) Does geography and/or host genetic diversity structure the microbiome community? (2) How is bacterial diversity and community assembly related to microeukaryotic diversity and community

assembly in the skin microbiome? (3) Do bacteria that affect *Bd* growth have predictable associations with microbiome eukaryotes?

Methods

Study system and field sampling

The focal species for this study is *Thoropa taophora,* a cycloramphid frog with a unique tolerance for coastal habitat that allows a wide distribution across the coastal Atlantic Forest of São Paulo State (Duryea et al. 2008). Adult *T. taophora* frogs were sampled from each of ten study populations: seven island populations and three coastal mainland populations (Fig. 3-5, Table 3-3). Genetic diversity is lower in island *T. taophora* populations relative to coastal mainland populations, both at neutral (microsatellite) loci (Duryea et al. 2015) as well as at the MHC IIB immunogenetic locus (Belasen et al. 2019). To examine how host genetics impact the skin microbiome diversity, skin swab samples were taken from the same individuals that were previously genotyped at MHC IIB (see Belasen et al. 2019). Frogs were thoroughly washed with sterile (autoclaved) distilled water and then swabbed on the ventral surface using standard protocols that minimize cross-contamination (Hyatt et al. 2007). DNA was extracted from swabs using a Qiagen DNeasy Blood and Tissue kit.

Microbiome sequencing and bioinformatic processing

Individual swab DNA extracts were barcoded, pooled and sequenced on the Illumina MiSeq platform (250 bp paired-end reads) in two assays: (1) barcoded 16S primers (515F and 806R) (Vences et al. 2016) were used to examine bacterial diversity; and (2) barcoded 18S v4 primers (TAReuk454FWD1 and TAReukREV3) (Stoeck et al. 2010) were used to examine

microeukaryote diversity. 16S libraries were constructed at the Universidade Estadual Paulista (BR) and sequenced at the Tufts University Core facility (USA) while 18S library preparation and sequencing was performed at the University of Michigan (USA). Negative (template-free) controls were run simultaneously with each sequencing library to ensure there was no contamination from PCR or sequencing reagents.

Sequences were quality-filtered and processed using the Quantitative Insights into Microbial Ecology (QIIME) MiSeq pipeline using default settings (Caporaso et al. 2010). As no mock community was included as a positive sequencing control, low abundance OTUs were filtered from the dataset using a conservative abundance threshold (<0.005% of all reads) (Bokulich et al. 2013). Sequences were clustered into operational taxonomic units (OTUs) using a 97% similarity threshold and compared against reference databases (rdp GreenGenes for 16S, Silva 97 for 18S) to assign taxonomy using the BLAST search algorithm. Chimeras were identified and filtered using UCHIME2 (Edgar 2016). For 18S data, sequences assigned to frog or other non-target non-microbial species (*e.g.*, Streptophyta) were filtered from the dataset. Sequences were rarefied to 1000 per sample for 18S data and 2000 per sample for 16S data. These values were selected based on visual examination of histograms and read accumulation curves constructed for all samples.

To infer ecological effects of bacteria on fungi and protists, bacterial OTU representative sequences from the *T. taophora* samples were compared against a reference database containing bacteria that were previously isolated from amphibian skin and evaluated for effects on *Bd* growth in co-culture experiments (Woodhams et al. 2015). The BLAST algorithm was implemented and an E-value threshold of E < 1e-20 was used for matches with the reference

database to categorize matching *T. taophora* skin bacteria as being *Bd* enhancing or inhibiting, or having no effect on *Bd* growth.

Data analysis

To evaluate overall patterns of microbiome alpha diversity, t-tests and Mann-Whitney U tests were performed to compare total observed eukaryotic or bacterial OTUs across site types (island vs. mainland) or MHC genotypes (homozygote vs. heterozygote) in SPSS (vrs. 22). Analyses of microbiome community structure (beta diversity) were conducted using Mantel tests of community dissimilarity vs. geographic distance or genetic distance (F_{ST}) implemented in the ade4 package of R (vrs. 1.7-11) (Dray and Dufour 2007; Chessel et al. 2004; Dray et al. 2007; R Core Team 2018).

To examine associations between microbial communities and geography or host frog MHC IIB genotype, data were statistically analyzed and visualized using packages implemented in Python (vrs. 2.7.13) and Matplotlib (Hunter 2007; van Rossum 1995). Associations between microbial communities and geography or frog MHC IIB genotype were determined by simulating an expected null distribution of host frog microbiomes. To create the null distribution, a two-column data table was created with column 1 being the site type (island or coastal) or MHC IIB genotype (heterozygous or homozygous) of a host frog and column 2 being one microbial OTU found on that frog. After the data table was populated for all frogs and microbes in the dataset, column 2 (microbial OTU) was held constant while column 1 (site type or frog genotype) was shuffled randomly. This was repeated 1000 times to create two sets of random

microbial occurrence distributions, one for analysis of microbial associations with site type and a second for analysis of microbial associations with host frog genotype.

Co-occurrence between microbial OTUs within and among domains (Bacteria vs. Eukaryotes) was analyzed with a third null distribution of microbial communities. Because of potential site effects on microbial presence and community structure (*e.g.*, some microbes only co-occur on frogs because the microbes themselves solely occur at the same subset of sites) and site-MHC IIB genotype interactions (as homozygotes and heterozygotes are not evenly distributed across sites or site types; Table 4-4), an expected null distribution of microbes accounting for site-specific presence/absence of each microbe was created. This null distribution of microbes was achieved through within-site randomization using MCMC edge swapping among frogs with the same MHC IIB genotype, a standard method for network datasets (Petersen 1891; Besag and Clifford 1989; Fosdick et al. 2018). Two microbe-frog pairs were randomly selected (each pair consisting of a single randomly selected microbial OTU found on a single randomly selected frog). Microbial OTUs were swapped between the selected frogs when three criteria were met: (1) the frogs were different individuals with the same MHC IIB genotype (either both homozygous or both heterozygous); (2) the OTUs were different from one another; and (3) neither frog already hosted the microbe it would receive via the swap. This method allows any configuration to be reached from any starting point, and allows for even sampling along all allowed states as forward and backward swaps are equally likely. Swapping was performed with 1000 repetitions for each frog-microbe pair (for microbial OTU swapping).

To test whether hypothesized bacterial effects on *Bd* extend to diverse microeukaryotic members of the microbiome, bacterial OTUs were binned according to their hypothesized ecological significance with regard to *Bd* (*Bd* inhibitory, *Bd* enhancing, or no effect on *Bd*;

Woodhams et al. 2015). Bacteria within each of these three categories were then compared with the third null distribution of microbial OTUs.

For all microbial association/co-occurrence analyses, the probability of non-random microbial association/co-occurrence (p) was calculated by comparing observed versus expected counts of microbial association/co-occurrence. P-values were evaluated at a significance level of $\alpha = 0.05$. Using the results of the tests of co-occurrences within and among all microbial taxa, network analyses were performed and visualized using SciPy (Hagberg and Schult 2008).

Results

Associations between geography, host genetics, and the skin microbiome

There were 845 microeukaryotic OTUs and 303 bacterial OTUs recovered across all samples after filtering and rarefaction. Mantel tests revealed significant positive associations between geographic distance and beta diversity in both eukaryotic and bacterial microbes, *i.e.*, populations that were geographically closer had significantly more similar microbiome community structure (eukaryotic taxa: $r = 0.18$, $p < 0.05$; bacterial taxa: $r = 0.40$, $p < 0.01$, Fig. 3-6). Neither MHC IIB genetic distance nor neutral genetic distance (from microsatellite data published in Duryea et al. 2015) were associated with microbiome community structure ($p > 0.1$ for all Mantel tests between 18S or 16S beta diversity and F_{ST} matrices for MHC IIB or microsatellites). Microeukaryotic diversity was positively correlated with bacterial diversity across all samples (number of OTUs; Spearman's $\rho = 0.25$, $p < 0.001$).

In the 16S bacterial dataset, Proteobacteria were dominant across all samples, both by number of OTUs and sequence reads (Fig. 3-7A&B). Proteobacteria also formed the core bacterial microbiome across samples (Fig. 3-7C). Among the eukaryotic microbiota, fungi were

dominant by both number of OTUs and sequence reads (Fig. 3-7D&E). No core group of eukaryotic microbiome taxa was recovered, though members of the Fungi were found in approximately 50% of samples (Fig. 3-7F). These common fungal OTUs included Ascomycota, Basidiomycota, and unidentified fungi.

Island frogs had fewer eukaryotic OTUs than mainland frogs (85.5 OTUs on average on islands vs. 110.5 on average on the mainland; Mann-Whitney test, $U = 2,604$, $p = 0.001$), but similar bacterial microbiome diversity. However, the composition of both bacteria and microeukaryotes varied by site type (Fig. 3-8A&B). Eight bacterial groups were significantly associated with site type: Cyanobacteria and Proteobacteria were statistically associated with coastal mainland sites, while six bacterial groups were statistically associated with island sites. Among the microeukaryotes, Rhizaria, Nucleariids, Ichthyosporeans, and Apusozoans were statistically associated with coastal sites while Fungi and Apicomplexans were statistically associated with island sites.

The diversity of microeukaryotes was significantly associated with MHC IIB diversity on the individual host level, with MHC IIB heterozygotes possessing a higher number of microeukaryote OTUs than MHC IIB heterozygotes (29.7 on heterozygotes vs. 16.6 on average on homozygotes; Mann-Whitney U, $U = 3,729.5$, $p < 0.01$). However, this result could be confounded by differences across island and mainland sites because both microbial community composition (Fig. 3-8A&B) and the number of MHC IIB heterozygotes and homozygotes vary across site types (Table 3-3). Therefore, the analysis of microeukaryote diversity among MHC genotypes was repeated on a subset of the data only including individuals from mainland sites. MHC IIB homozygotes still possessed significantly fewer microeukaryote OTUs than heterozygotes when only mainland frogs were included in the analysis (19.2 on average on

homozygotes vs. 39.1 on heterozygotes; Mann-Whitney U, $U = 681.5$, $p < 0.01$). The number of bacterial OTUs was not significantly different between MHC IIB homozygotes and heterozygotes (t-test, $p > 0.05$).

Microbiome community composition varied between MHC IIB heterozygotes and homozygotes for both bacteria and microeukaryotes when compared with null expectations based on genotype randomizations, with heterozygotes hosting significantly more unidentified Bacteria, Bacteroidetes, and Firmicutes, but fewer Actinobacteria and Proteobacteria OTUs than homozygotes (Fig. 3-8C). In terms of microeukaryotes, MHC IIB heterozygotes hosted significantly more OTUs belonging to the Ciliates, Rhizaria, and Stramenopiles, but significantly fewer Fungi and Algae OTUs than homozygotes (Fig. 3-8D).

Microbial networks within and among domains

Networks were first constructed for bacteria only and microeukaryotes only based on tests of co-occurrence between OTUs within and among taxonomic groups (Fig. 3-S11). A dominant bacterial network assembled that consisted of Firmicutes and Bacteroidetes at the center with connections to Fusobacteria, Spirochaetes, Verrumicrobia, Deferribacteres, and unidentified bacteria (Fig. 3-S12). A second group was composed of Gemmatimonadetes and Cyanobacteria. Actinobacteria and Proteobacteria did not form network connections with any other groups, although strong connections were formed among OTUs within the Proteobacteria. Only one small microeukaryotic network was formed that consisted of five taxonomic groups: Alveolates were at the center of the network and formed connections with Apicomplexans, Rhizaria, and unidentified microeukaryotes, which in turn connected with Nucleariids (Fig. 3-

S13). The remaining 16 microeukaryote groups remained unconnected to the network, though there were strong connections among OTUs within the Algae.

The construction of a network between bacteria and microeukaryotes revealed a greater number of connections among groups than either the bacterial or microeukaryotic network (Fig. 3-9). A majority of taxa (12/18) that had formed no connections in the bacteria-only and microeukaryote-only networks formed connections with other taxa in the overall microbial network. Specifically, these newly connected taxa included the two previously unconnected bacterial groups, Actinobacteria and Proteobacteria, and 10/16 previously unconnected microeukaryote groups.

Associations between bacteria reported to inhibit, enhance, or have no effect on Bd growth with microbiome eukaryotes

To determine whether bacteria previously reported to affect Bd growth had corresponding associations with other fungi and protists in the skin microbiome, we compared our set of bacterial OTU representative sequences against a published FASTA of bacterial OTUs that were previously isolated from a diversity of live amphibians and tested against Bd in co-culture inhibition experiments (Woodhams et al., 2015). The bacterial OTUs in the database that matched OTUs we recovered included members of the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Fig. 3-S14).

Tests of co-occurrence between eukaryote groups and matched bacterial OTUs revealed that enhancing, inhibitory, and no effect do not generally reflect the associations of these bacteria with microeukaryotes, both when examining solely fungi and when examining other microeukaryote taxa (Fig. 3-10). Bacteria previously found to enhance Bd growth were

negatively associated with the Ascomycota and Basidiomycota fungi, and also showed strong but marginally non-significant positive associations with the Chytridiomycota and Choanoflagellates. Bd inhibitory bacteria showed significant positive associations with the Cryptomycota fungi and Choanoflagellates, and significant negative associations with the Basidiomycota fungi and other unidentified fungi. Finally, bacteria that were previously found to have no effect on Bd were positively associated with the Ascomycota, Choanoflagellates, and Rhizaria, and showed strongly but marginally non-significant positive associations with Algae, Amoebae, Ciliates, and Ichthyosporeans.

Discussion

Amphibian skin microbiomes exhibited high microeukaryote diversity and were dominated by Proteobacteria

In this study, we examined amphibian skin microbiome structure and diversity with respect to geography and host genetics. We sequenced both bacteria and microeukaryotes to compare associations between geographic and genetic factors and microbial diversity and to evaluate patterns of microbial co-occurrence across domains. Microeukaryotes in T. taophora skin microbiomes were apparently more diverse than bacteria, totaling 845 OTUs compared with only 303 bacterial OTUs. The diversity of microeukaryotes we recovered is higher than that previously reported from wild frogs (e.g., 255 OTUs following rarefaction on Rana cascadae; Kueneman et al. 2017). In contrast, the level of bacterial diversity we recovered is lower than has been previously reported (e.g., ~600 OTUs on Rana italica in Federici et al. 2015). However, our recovery of bacteria from 11 phyla is within the range of taxonomic diversity previously recovered from amphibian skin, with for example 10-18 bacterial phyla reported from three

species (McKenzie et al. 2012). Our analysis showed that total microeukaryotic and bacterial diversity were positively correlated across all samples, which is a novel finding to our knowledge. It seems unlikely that this pattern is an artifact of sequencing: different MiSeq runs (and different research facilities) were used to sequence microeukaryotes and bacteria.

Proteobacteria were the most dominant bacterial phylum on T. taophora skin across all study populations, in terms of both percent of OTUs and percent of reads. This is similar to findings from bacterial microbiome studies of other tropical post-metamorphic anurans (Abarca et al. 2018; Belden et al. 2015; Bletz et al. 2017; Varela et al. 2018). One hypothesis for the dominance of Proteobacteria on frog skin is that many members of the Proteobacteria produce anti-Bd metabolites (M. H. Becker et al. 2015; Brucker et al. 2008). The presence of a high number of Proteobacteria on T. taophora skin could hypothetically be a factor contributing to the low apparent susceptibility to Bd we previously observed in this species (Belasen et al. 2019). It is important to note however that the present study is correlative; without experimental manipulations it is difficult to pinpoint which factors (e.g., the physiology of the skin, mucosal biochemistry, host-microbial evolutionary processes, or interactions with the saline coastal environment) are responsible for the overwhelming dominance of Proteobacteria on anuran skin.

Although bacteria were less diverse than microeukaryotes in our samples, bacteria could nevertheless dominate the skin microbiome according to biomass, which we did not quantify in our study. Sequence reads are sometimes used as a proxy for relative abundance, but this has been shown to be an unreliable measure due to known sequencing biases among microbial taxa (Amend et al. 2010). We acknowledge the likelihood that taxa representing fewer OTUs (i.e., bacteria) could represent a higher proportion of microbial biomass, and that this should be considered in interpretations of our results. Future research to address the relationship between

microbial diversity and abundance should utilize high-throughput sequencing alongside quantitative analyses, for example quantitative PCR.

Microbiome structure varied with geography and host immunogenetics

Geography was a significant factor in microbiome structure (beta diversity) for both bacterial and eukaryotic microbes. However, microbiome structure was not associated with genetic structure of populations at either neutral genetic markers or the MHC IIB immunogenetic locus. These results differ from a previous study on the frog Amietia hymenopus, which showed opposite patterns: there were no geographic effects on amphibian skin microbiome structure, but there was a significant association with population genetic structure (Griffiths et al. 2018). One explanation for the discrepancy between our results and the results from A. hymenopus (barring factors related to host identity) may be that geographic structure in the host-associated microbial community may be scale-dependent: our study spans a larger geographic area (~100 km compared with ~4 km in Griffiths et al. 2018). In addition, our study populations represent a set of connected mainland populations contrasted with a set of island populations that have been isolated for 12,000-20,000 years. The lack of association with genetic differentiation in our populations may be due to this relatively long period of divergence, or to isolation between island sites resulting in different environmental availability of microbes.

Microeukaryote diversity was associated with host genetic diversity, with genetically impoverished island populations possessing lower microeukaryotic diversity (observed OTUs) relative to coastal mainland populations (85.5 on average on islands vs. 110.5 on average in costal sites). This difference in microbiome diversity could be due to a number of factors, including less favorable environments or lower rates of host contact (i.e., microbial transmission)

on islands compared with coastal sites. However, MHC IIB individual-level diversity (heterozygosity) was positively associated with microeukaryotic diversity even when only coastal populations were analyzed (19.2 on average on coastal homozygotes vs. 39.1 on coastal heterozygotes). Taken together, these results imply that genetic diversity and/or MHC IIB genotype plays a significant role in determining microbiome diversity.

In addition to microbiome diversity, microbiome structure also varied across site types and MHC IIB genotypes. Variation in microbiome structure among site types could be parsimoniously explained by variation in environmental filtering in coastal vs. island sites. However, these differences may also be driven by island isolation favoring longer-dispersing microbes, or alternatively by host genetic factors. The variation in microbiome structure across MHC IIB genotypes, although weaker than the variation due to site type, may be a clearer example of associations between endogenous host factors and the microbiome. Although MHC genes are thought to be primarily involved in pathogen resistance, laboratory and field studies have shown that MHC genotype and allelic composition can impact amphibian host-associated microbial assemblages (Barribeau et al. 2012; Hernández-Gómez et al. 2018). These studies corroborate our finding that microbiome structure and diversity are influenced by MHC genotype, and suggest that immune mechanisms conferred by MHC genes may influence the assembly of the overall microbiome.

Cross-Domain co-occurrence in the amphibian skin microbiome network

Our network analyses revealed a number of notable patterns. The bacterial network consisted of a major and minor group, and the majority of microeukaryote groups did not form significant connections in the eukaryote-only microbial network. However, the overall microbial

network revealed that a number of microbial groups exhibit cross-Domain co-occurrence (i.e., between bacteria and eukaryotes): a majority of previously unconnected microeukaryote groups (10/16) and both previously unconnected bacterial groups became connected in the overall microbiome network.

One important implication of this result is that ecological interactions exist between microbiome bacteria and eukaryotes that may significantly impact microbiome assembly. An alternative explanation is that bacteria and eukaryotes that co-occur experience co-filtering via specific host, environmental, or other exogenous factors. It is unclear how widespread these cross-Domain microbiome connections are across amphibian host species. Previous studies that have examined both bacteria and microeukaryotes on amphibian skin have mainly focused on taxon-specific associations, for example between Bd-inhibitory bacteria and fungi (Kueneman, Woodhams, Van Treuren, et al. 2016), and between Bd and either bacteria or microeukaryotes (Kueneman et al. 2017). To our knowledge, ours is the first study to demonstrate these cross-Domain network connections in the amphibian skin microbiome.

Bd inhibitory and enhancing bacteria have variable effects on microbiome fungi and protists

Our dataset included a number of bacteria previously shown to inhibit Bd, which have been generally termed “antifungal” in the literature (Vences et al. 2016) although empirical support for this broad designation comes from only a single study (Kueneman, Woodhams, Van Treuren, et al. 2016). Bacteria with previously demonstrated effects on Bd growth did not show general patterns with T. taophora skin microbiome eukaryotes. Although bacteria previously found to enhance Bd growth were positively associated with the Chytridiomycota (the phylum containing Bd, although Bd was not present in our 18S dataset), these bacteria were negatively

associated with Ascomycota and Basidiomycota fungi. Perhaps more critical are the relationships with Bd inhibitory bacteria, as these bacteria have been proposed for use in probiotic treatments for the management of Bd infections (Bletz et al. 2013; Walke and Belden 2016). Bd inhibitory bacteria showed weak positive associations with Cryptomycota fungi and significant negative associations with Basidiomycota fungi and other unidentified fungi in the T. taophora skin microbiome. Bd-inhibitory bacteria were also positively associated with Choanoflagellates, and showed strong though non-significant positive associations with the Zoopagomycota and Ichthyosporea.

These results suggest that probiotic treatments in wild populations may have unintended consequences for microbiome stability. According to our analyses, specific attempts to increase Bd inhibiting bacteria and/or reduce Bd enhancing bacteria in wild frog populations could have unwanted effects, such as potentially reducing beneficial fungi in the Dikarya (Ascomycota and Basidiomycota; Kearns et al. 2017), and augmenting poorly studied parasites such as Ichthyosporea protists (Rowley et al. 2013) and fungi including Ascomycota and Zoopagomycota (Badali et al. 2010; Seyedmousavi et al. 2015). These hypothetical effects warrant further study, for example through culture-based or in vivo challenges between proposed probiotic bacteria and these parasitic microeukaryotes.

Limitations and future research priorities

Taken together with recent studies (Kearns et al. 2017; Kueneman et al. 2017), our results suggest that focusing only on bacteria provides an incomplete picture of the host-associated microbiome. Granted, as in many other amphibian microbiome studies (Vences et al. 2016; Kueneman, Woodhams, Van Treuren, et al. 2016) our study presents microbes at a relatively

coarse phylogenetic resolution (generally phylum level). Very large differences in ecology and environmental requirements likely exist between OTUs within these higher-order classification levels, thus the patterns we detected may change with higher-resolution taxonomic data. With advancing technology allowing for increased sequence length (e.g., using third-generation sequencing), more efficient microbiome analysis pipelines, and well-curated reference sequence databases, future cross-Domain microbiome research at higher taxonomic resolution should be prioritized.

Our results imply that host immunogenes play a role in structuring the amphibian skin microbiome. Furthermore, our network analyses suggest that there may be important interactions between bacteria and microeukaryotes that have been missed by previous microbiome studies focusing on only one of these Domains. Given the widespread use of bacterial probiotic treatments in humans as well as in domesticated and wild animals (Gram et al. 1999; Ghadban 2002; Cheng et al. 2017), future studies should prioritize advancing our understanding of interactions between microbiome bacteria and eukaryotes.

Tables

Table 3-3: Sampling site data.
Sample size is the number of frogs collected at each site. MHC IIB heterozygosity is the observed heterozygosity, or number of heterozygotes over the total individuals genotyped from each population. Site locations are shown in Figure 2-1.

Site name	Site code	Site type	Latitude	Longitude	Sample size	MHC IIB heterozygosity (H_o)
As Ilhas	AI	Island	-23.789276	-45.711507	4	0
Couve Sul	CS	Island	-23.800899	-45.721672	7	0.43
Couves Norte	CN	Island	-23.422075	-44.854066	30	0.43
Gatos	GA	Island	-23.805592	-45.670011	3	0
Porcos Pequena	PP	Island	-23.377864	-44.904266	20	0
Prumirim	PR	Island	-23.384791	-44.945678	22	0.09
Tamandua	TA	Island	-23.597168	-45.288857	25	0
Barra do Una	BU	Coastal	-23.761536	-45.770697	20	0
Sununga	SU	Coastal	-23.508867	-45.133827	20	0.7
Toque Toque	TT	Coastal	-23.835912	-45.509922	24	0.51

Figures

Figure 3-5: Locations of Thoropa taophora sampling sites on the coast and islands of São Paulo, Brazil.
Red circles indicate islands and white triangles indicate coastal (mainland) sites. Site information including full site names, two-letter codes, latitude/longitude, and frog sample size can be found in Table S1.

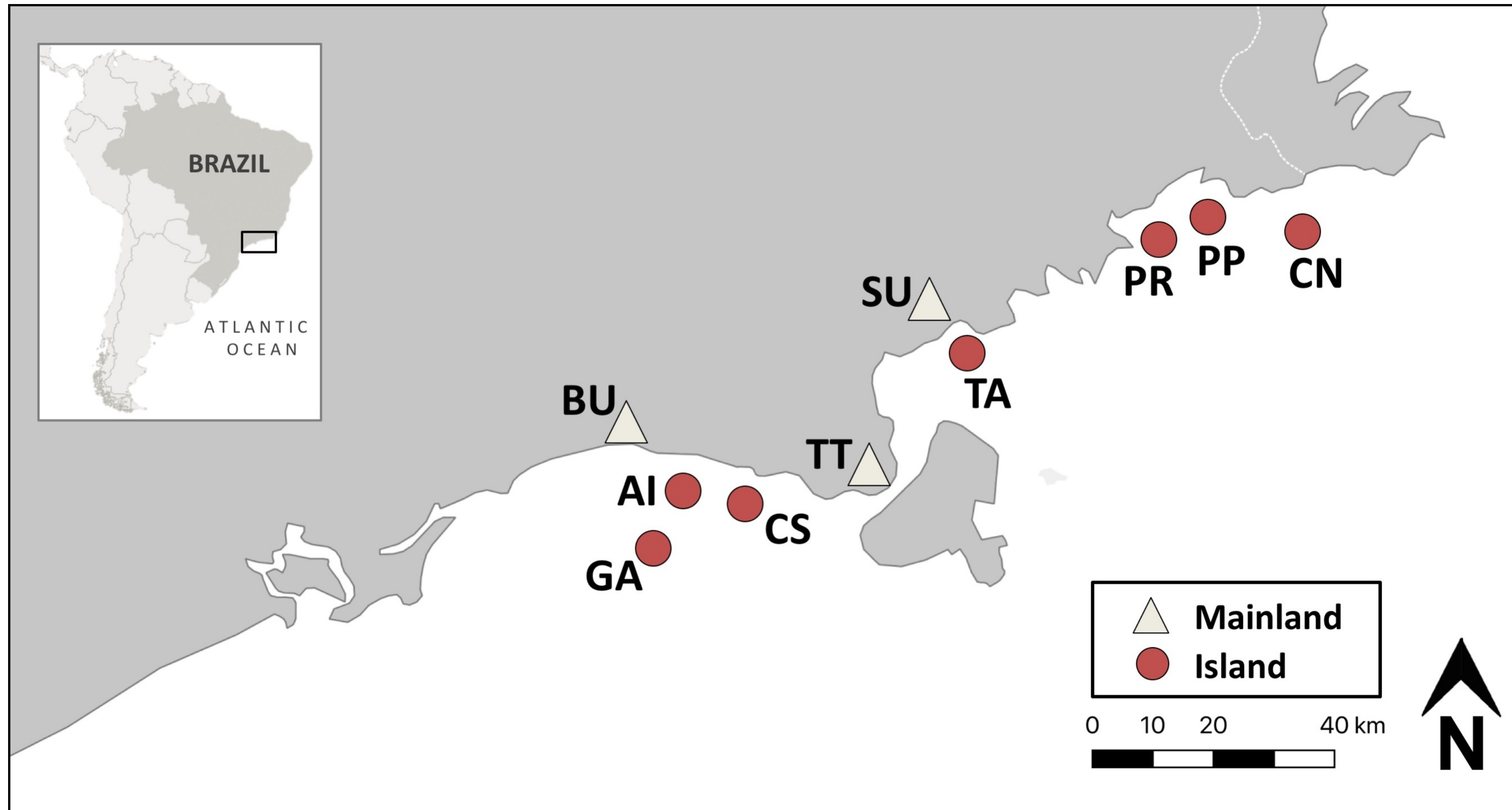


Figure 3-6: Microbiome dissimilarity and geographic distance.
Geographic distance is significantly associated with microbiome community dissimilarity (beta diversity) for Bacteria (right) and Eukaryotes (right) found in the *Thoropa taophora* skin microbiome. Blue dashed lines indicate significant positive relationships supported by Mantel correlations ($p < 0.05$).

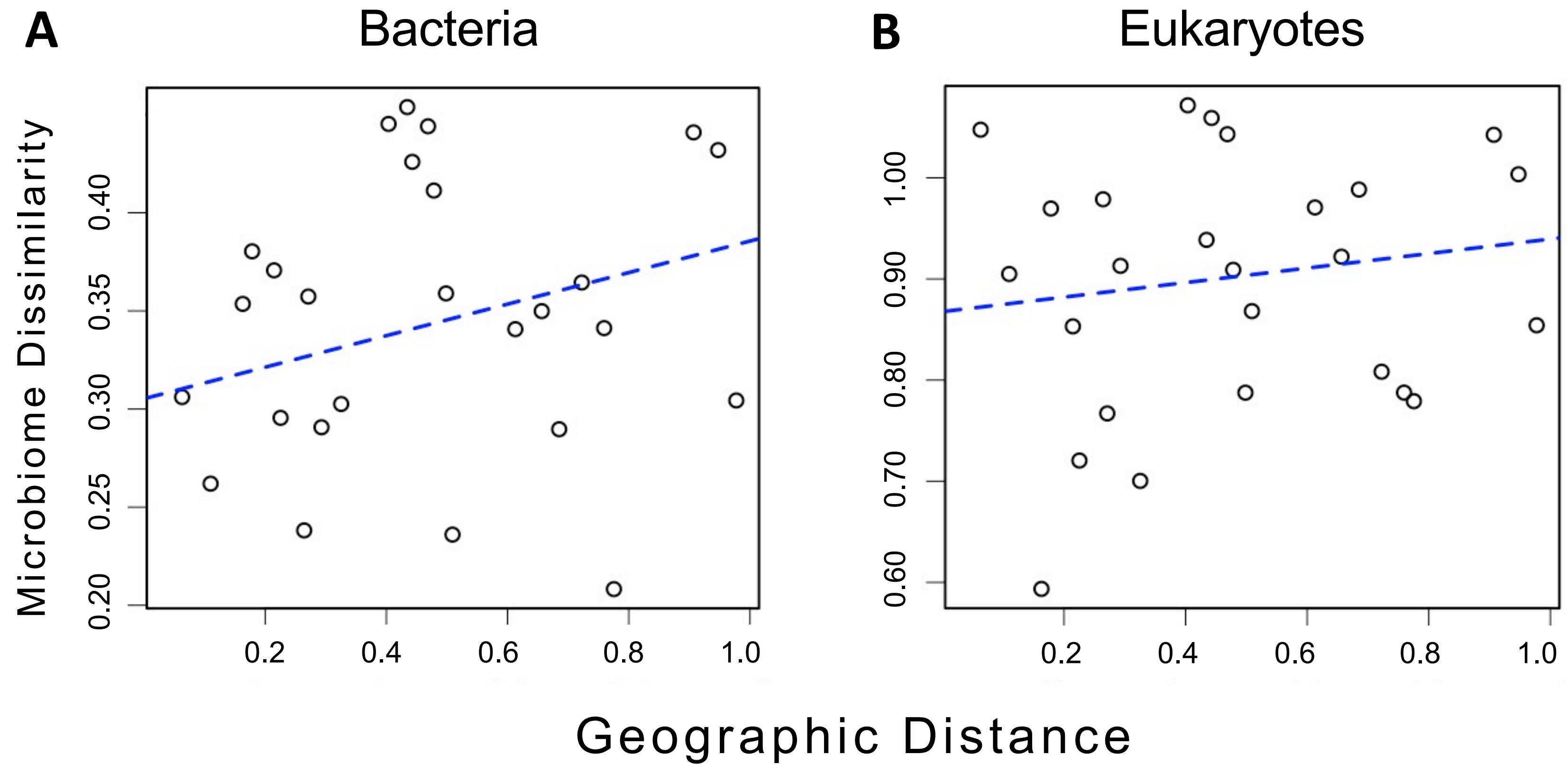


Figure 3-7: Distribution of microbiome taxonomic diversity across study sites and individuals.
Bacteria are shown in A-C and eukaryotes in D-F. Stacked barplots show the relative abundance of microbial taxa by number of OTUs (A & D) and reads (B & E). Frequency histograms (C & F) show the percent of OTUs across populations. Bolded site codes indicate island sites.

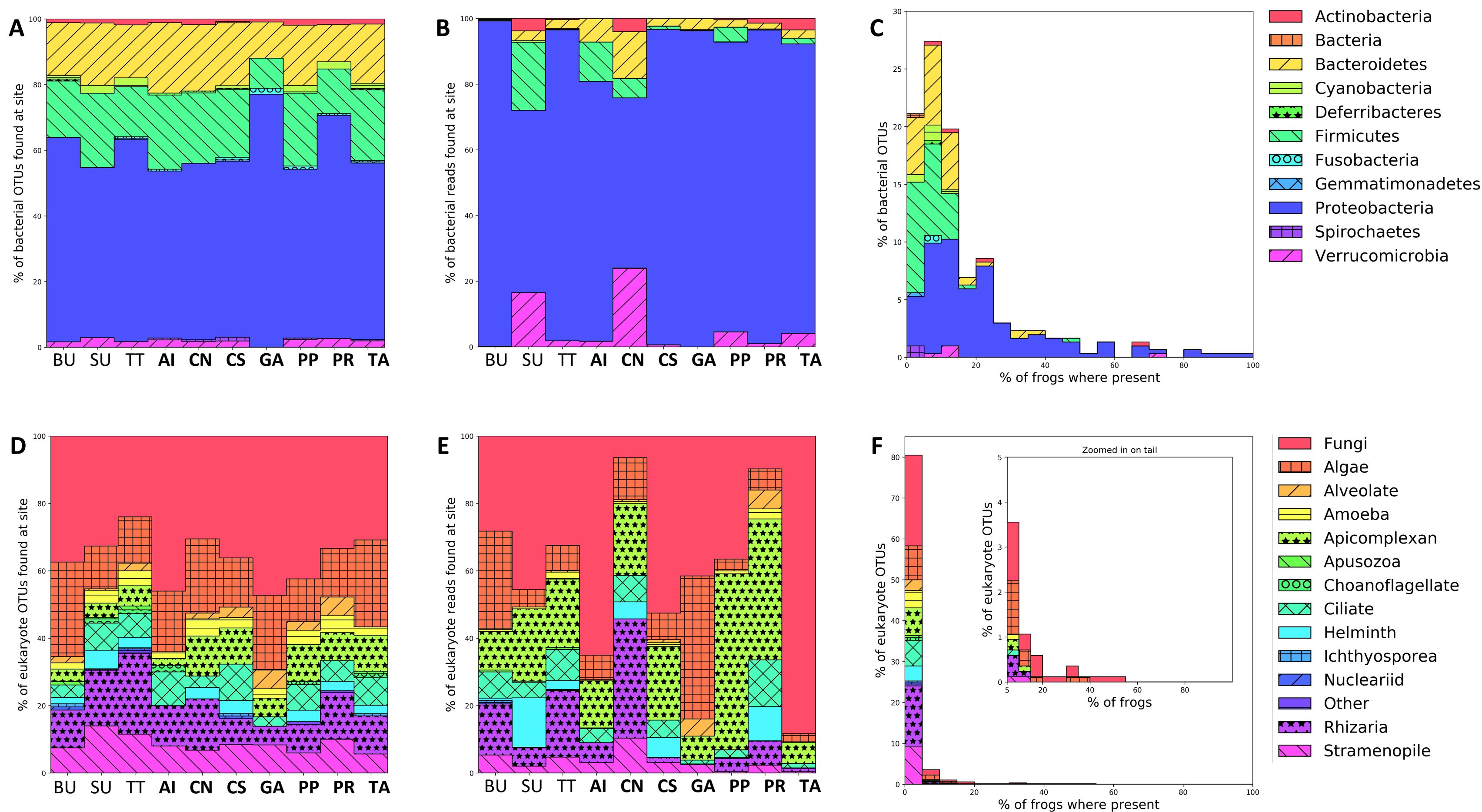


Figure 3-8: Heat maps of microbes across sites and host MHC IIB genotype.
Bacteria are shown in A and C, eukaryotes are shown in B and D. Associations between microbial OTUs and site type (coastal/mainland vs. island) are shown in A and B, and associations between microbial OTUs and frog MHC IIB genotype (heterozygous vs. homozygous) are shown in C and D. The more saturated the red, the stronger the positive association between taxa and site type or genotype, and the more saturated the blue, the stronger the negative association. To determine associations, actual distribution of microbes was compared to 1000 randomly generated microbiomes within each site. Black dots represent significant deviation from random expectations with $p < 0.05$, and white stars represent $p < 0.01$.

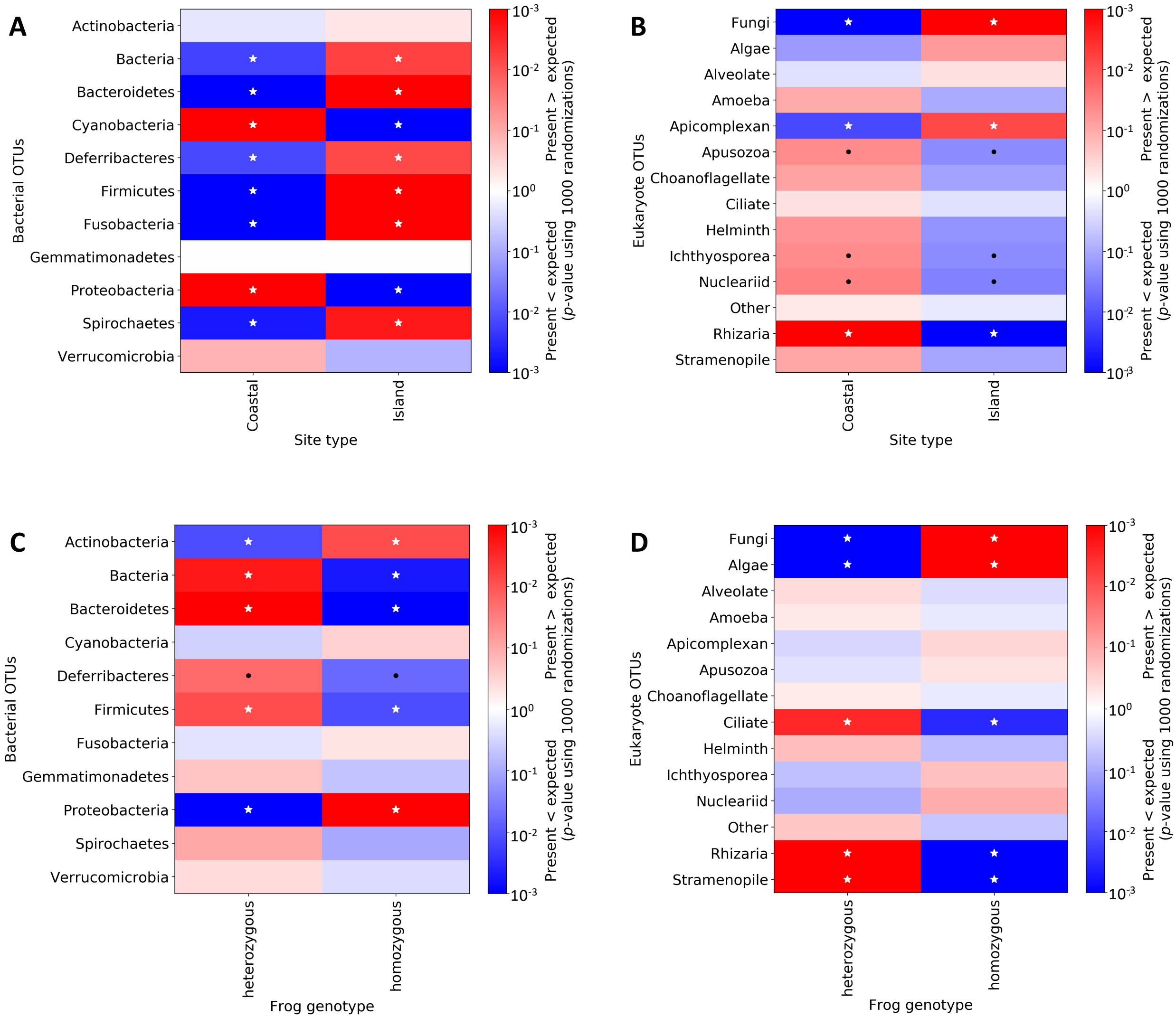


Figure 3-9: The bacterial-eukaryotic microbiome network and taxon co-occurrence associations.
Microbiome network showing associations between bacterial and microeukaryotic OTUs. Bacteria are triangles, Fungi are squares, and Protists are circles. The size of the symbol shows the relative abundance of each taxon. Stronger associations are indicated by thicker/darker network branches between symbols (associations of OTUs among taxa) or circles around symbols (associations of OTUs within a taxon). Branches and circles are significant at $p < 0.001$.

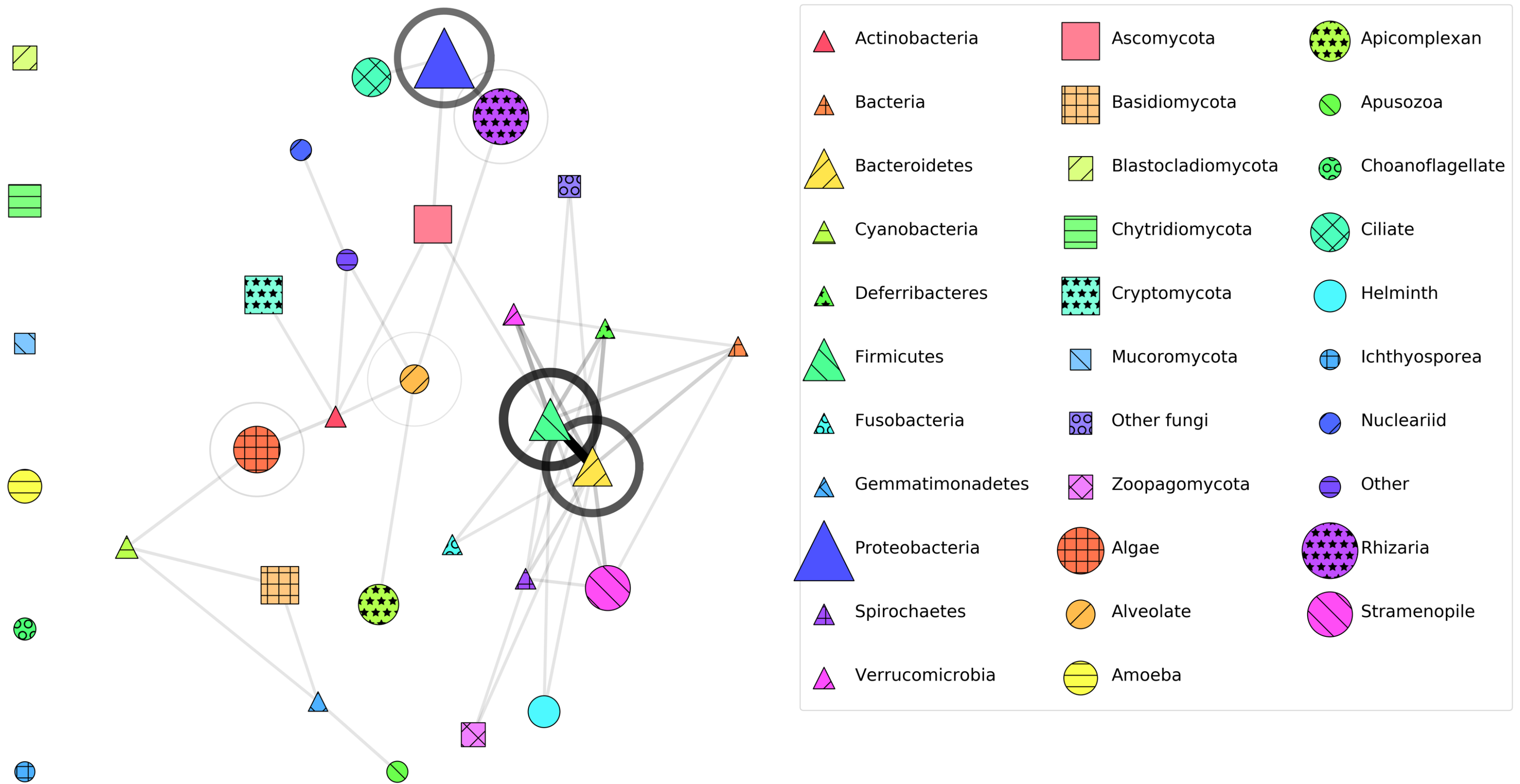
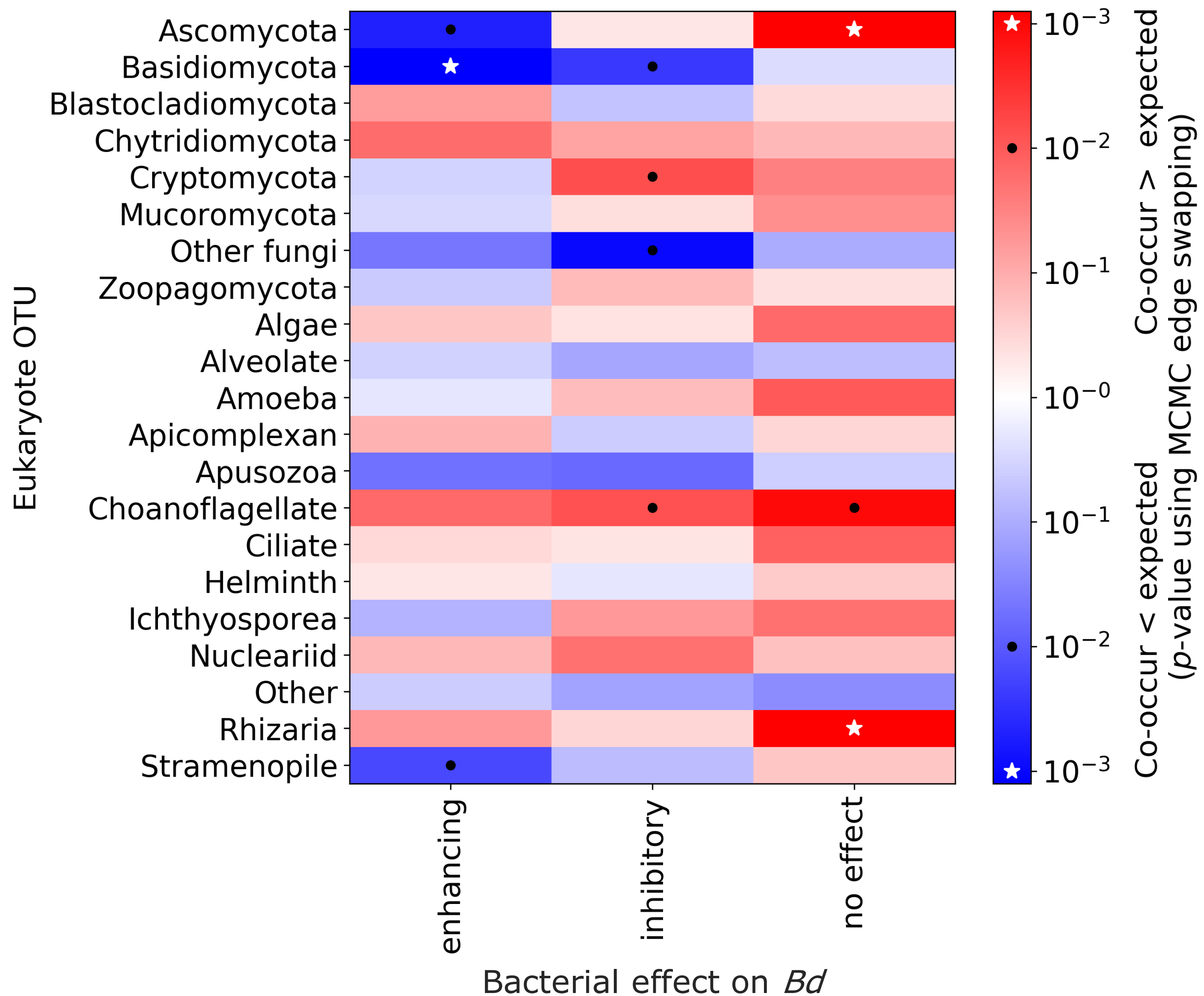


Figure 3-10: Heat map of microeukaryote co-occurrence with bacterial OTUs found in *T. taophora* skin swabs.
Bacteria are binned into groups corresponding to "Effects on Bd" based on matches to bacterial OTUs categorized by Woodhams et al. (2015). The more saturated the red, the stronger the positive association between two taxa, and the more saturated the blue, the stronger the negative association. Black dots represent a significant deviation from random co-occurrence with $p < 0.05$, and white stars represent $p < 0.01$.



Supplemental Figures

Figure 3-S11: Heat map depicting co-occurrence between bacteria and microeukaryotes.
The more intense the red, the stronger the positive association between two taxa, and the more intense the blue, the stronger the negative association. Black dots represent a significant deviation from randomly generated microbiomes (1000 repetitions per site) with $p < 0.01$, and white stars represent $p < 0.001$.

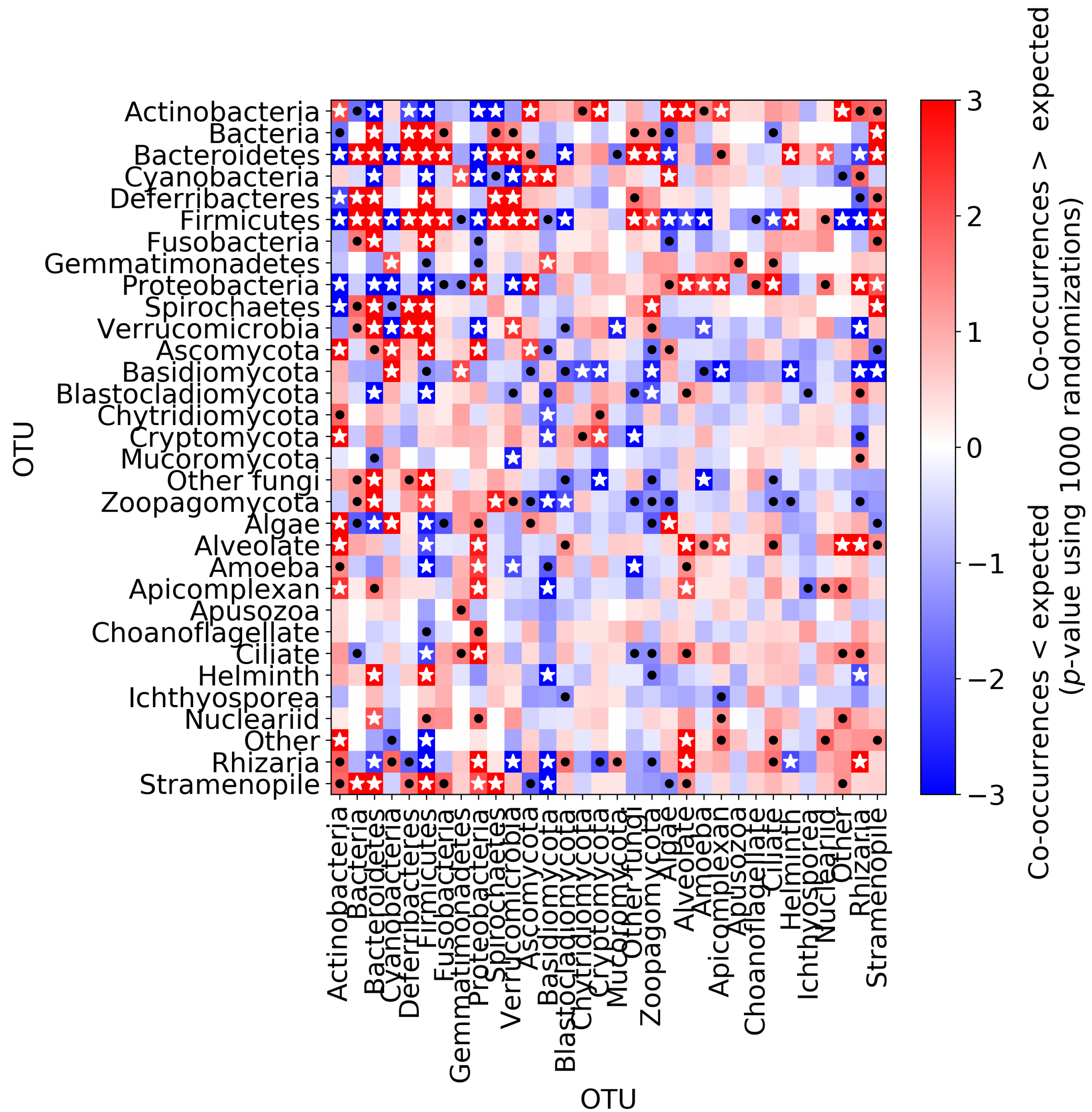


Figure 3-S12: Microbiome network showing associations between bacterial OTUs.
Connections shown in network below are significantly different from randomly generated microbiomes (1000 repetitions per site) at a level of $p < 0.001$. A circle around a symbol indicates strong associations between taxa within the group. The size of the symbol shows the relative abundance of each taxon.

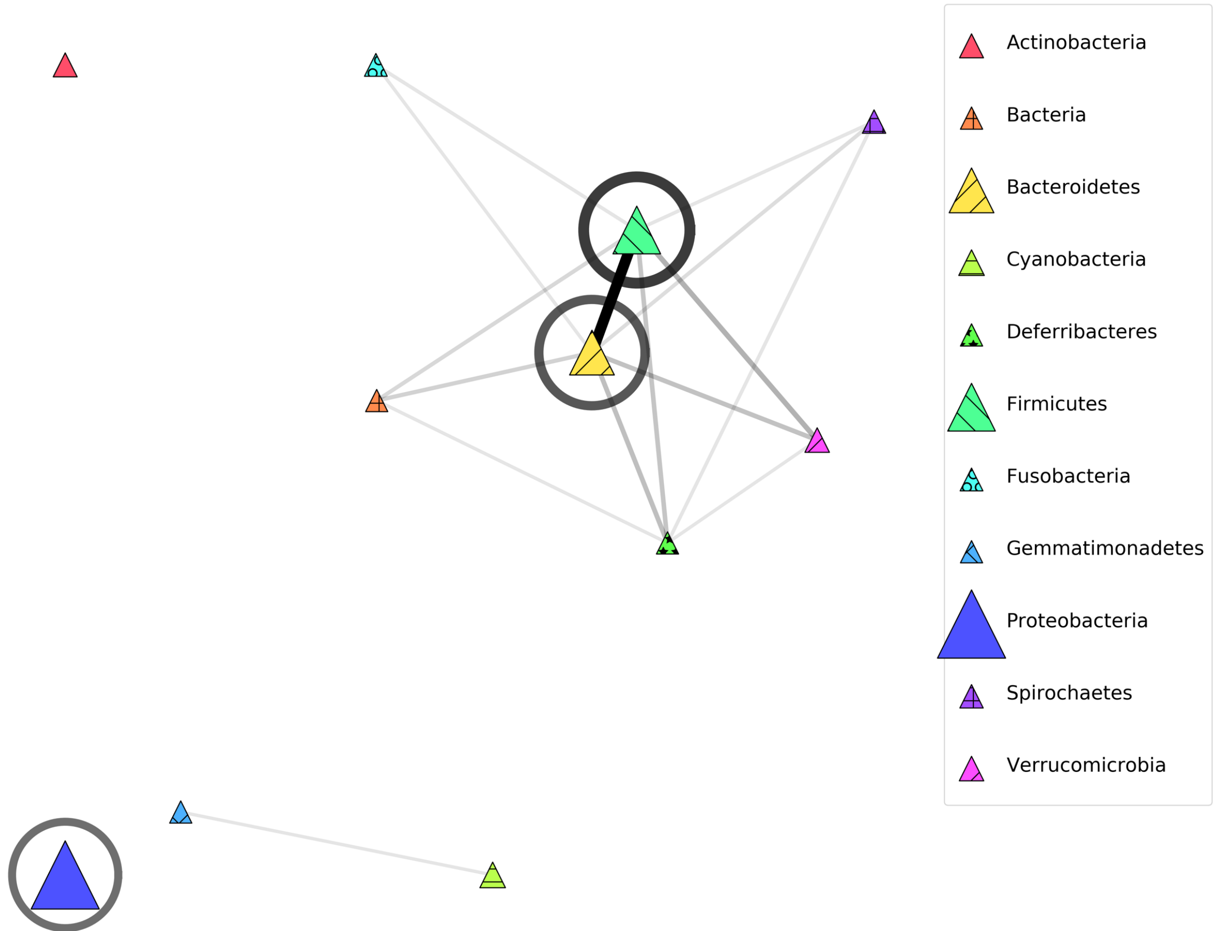


Figure 3-S13: Microbiome network showing associations between microeukaryotic OTUs. Connections shown in network below are significantly different from randomly generated microbiomes (1000 repetitions per site) at a level of $p < 0.001$. A circle around a symbol indicates strong associations between taxa within the group. Fungi are squares, and other eukaryotes are circles. The size of the symbol shows the relative abundance of each taxon.

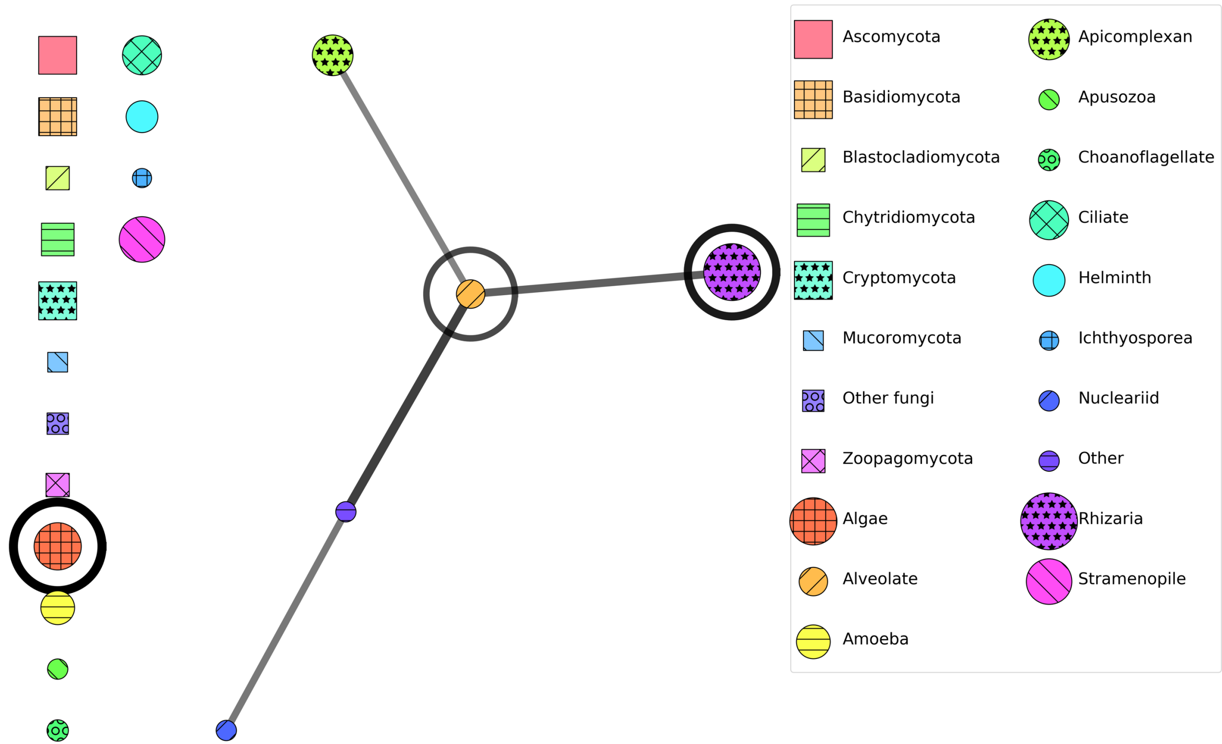
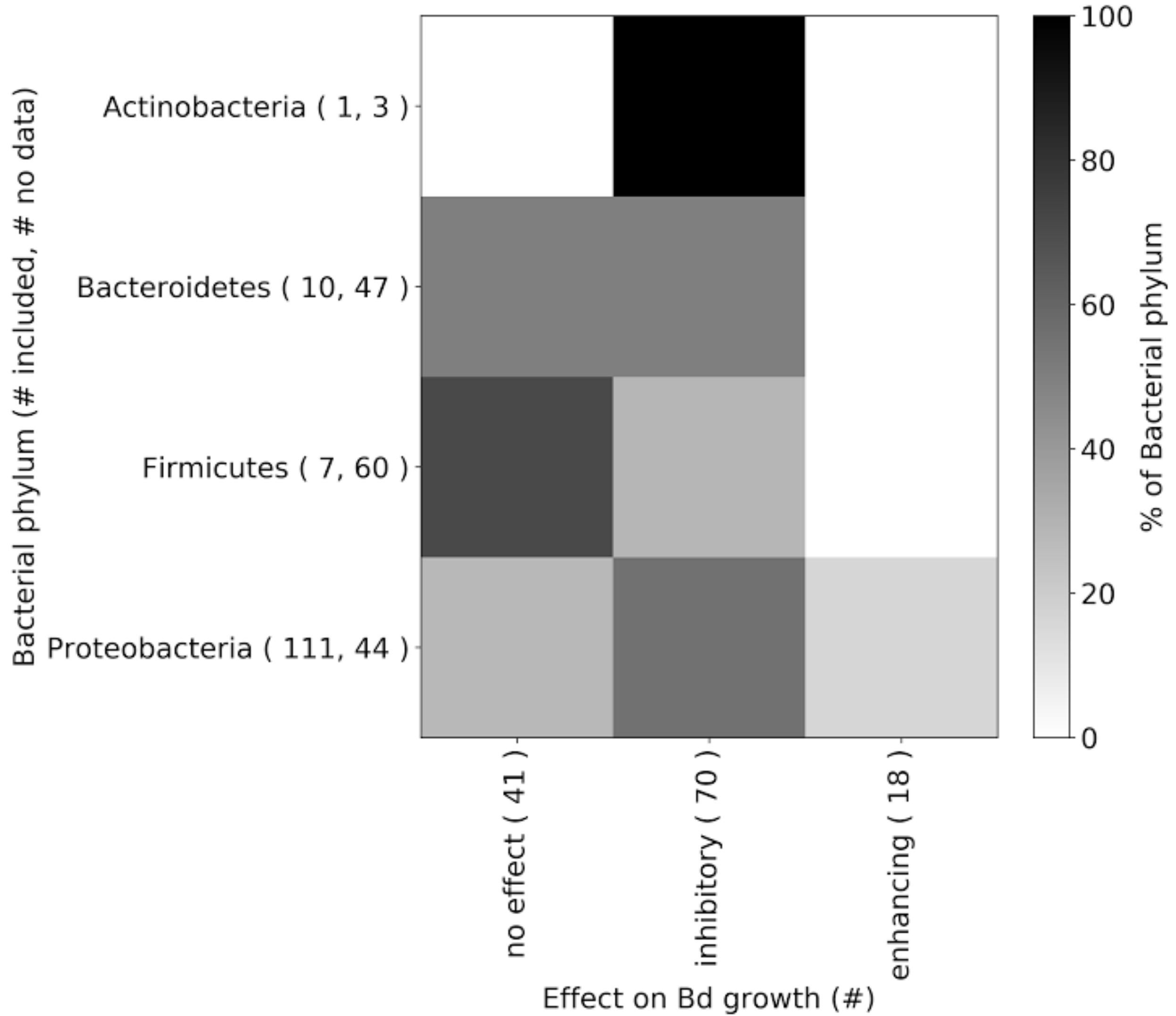


Figure 3-S14: OTUs in bacterial phyla from *T. taophora* skin swabs that matched representative sequences from Woodhams et al. (2015).
In parentheses next to the bacterial phyla on the y-axis are the number that matched the database followed by the ones that did not have a match. In parentheses on the x-axis are the numbers of OTUs in total found in each group.



Chapter 4: The Effects of Habitat Modification in Frogs of the Brazilian Atlantic Forest Depend On Land-Use Intensity and Frog Species Ecology[3]

Abstract

Habitat modification threatens global biodiversity. Habitat modification includes fragmentation, degradation, and loss, all of which contribute to wildlife extinctions. Wildlife populations that survive in fragmented and degraded habitats may go extinct in the future due to genetic erosion that grows more severe over time due to generations of inbreeding and genetic drift. However, it remains to be understood how the effects of habitat modifications on genetic diversity vary across land-use types (forest fragments in a matrix of intensive agriculture vs. continuous rustic agroforests) or divergent species ecologies (high dispersing vs. low dispersing, habitat specialists vs. generalists). In this study, we examined the impacts of habitat fragmentation on frog genetic diversity in the Brazilian Atlantic Forest across a range of land-use types and species ecologies. We collected tissue samples from six frog species that vary in ecology, and compared populations in forest fragments to those in continuous forests that were either preserved forests or managed, rustic agroforests. From these tissue samples we produced genomic datasets using a reduced representation library approach (double-digest restriction site-associated DNA sequencing) to quantify genetic diversity and genetic isolation. We found that while only habitat generalist frogs exhibited reduced genetic diversity with fragmentation, habitat specialists showed signatures of genetic isolation due to fragmentation. Contrary to

[3] This chapter will be submitted for publication with the following co-authors: C. Guilherme Becker, L. Felipe Toledo, and Timothy Y. James.

expectations, forest specialist frogs exhibited higher genetic diversity in fragments relative to continuous forest. Frog genetic diversity in continuous modified agroforests was either comparable or higher than frog genetic diversity in continuous preserved forests. Together our results suggest that the impacts of landscape modification vary with both species ecology and land-use context, and that forest fragments and modified agroforests constitute areas of high conservation value.

Introduction

Biodiversity loss threatens human health and sustainability, and is largely attributed to anthropogenic activities (Wake and Vredenburg 2008). Habitat fragmentation*,* the division and isolation of natural areas, is arguably the most widespread anthropogenic driver of wildlife declines. In particular, tropical ecosystems contain a large proportion of natural habitats fragmented by intensive agriculture, which can pose a significant barrier to wildlife dispersal and population integrity (Perfecto and Vandermeer 2010). Tropical areas contain the highest proportion of biodiversity on earth and provision vital global-scale ecosystem services (Naidoo et al. 2008). Therefore, among all anthropogenic contributors to biodiversity loss, habitat fragmentation likely poses the greatest threat to global biodiversity and sustainability.

Habitat fragmentation leads to the loss of wildlife species (Almeida-Gomes et al. 2016) due to habitat loss as well as changes to abiotic and biotic factors within habitat fragments. Habitat fragments exhibit increased variability in temperature and humidity (Broadbent et al. 2008), altered habitat structure and habitat quality (Lôbo et al. 2011; Marsh and Pearman 1997; Hillers et al. 2008), and shifts in community assembly that alter biotic interactions (Boulinier et al. 2001; Leal et al. 2012). When fragments are separated by an aversive matrix such as intensive

agriculture, surviving animal taxa may experience reduced or total loss of dispersal (Watling and Donnelly 2007; Johansson et al. 2005). Loss of dispersal compounded with reduced population size in fragments leads to erosion of genetic diversity (Lesbarrères et al. 2002; Andersen et al. 2004) due to the combination of increased inbreeding and genetic drift (Johansson et al. 2007; Frankham et al. 2002). This loss of diversity in turn can reduce wildlife fitness and resilience against additional stressors (Johansson et al. 2007; Allentoft and O'Brien 2010).

However, the impacts of fragmentation on animal taxa can vary based on landscape context (*e.g.*, interfragment matrix quality) and species ecology (reviewed in Keyghobadi 2007 and Tabarelli et al. 2010). Wildlife species richness and genetic diversity can be maintained when farmers adopt less intensive agricultural practices (Johansson et al. 2005) or use rustic farming systems such as shade coffee (Pineda and Halffter 2004; Perfecto and Vandermeer 2010). The impacts of fragmentation can also vary according to the sensitivity of a given species' ecology to environmental changes. Previous multi-species studies have shown that habitat generalist species tend to survive and dominate in fragmented habitats, while habitat specialists tend to experience population declines (Kolozsvary and Swihart 1999; Hillers et al. 2008; Leal et al. 2012; Keyghobadi 2007; Tabarelli et al. 2010; Harrison and Bruna 2012). In some cases, habitat generalists can experience increased abundance, species richness, and/or genetic diversity following fragmentation (Gascon et al. 1999; Keyghobadi 2007). However, the variation in genetic effects of fragmentation in habitat generalists versus specialists remains little explored.

Among vertebrates, amphibians are particularly sensitive to environmental changes and habitat modification and have experienced global declines as a result of these processes (Cushman 2006). Because amphibians occupy central positions in food-chains and serve critical roles in nutrient cycling between aquatic and terrestrial habitats, the loss of amphibians can have

ecosystem-wide, long-lasting, and irreversible consequences (Rantala et al. 2015). Anurans (*i.e.*, frogs and toads) may be particularly susceptible to genetic diversity loss due to fragmentation. Anurans with typical explosive breeding ecologies (where many individuals simultaneously reproduce at the same breeding site) have naturally low effective population sizes relative to census population size, because few individuals produce offspring that survive to reproductive maturity (Allentoft and O'Brien 2010). A number of studies have shown the predicted negative impacts of fragmentation on amphibian richness and abundance (Funk et al. 2005; Bell and Donnelly 2006; Bickford et al. 2010; Hillers et al. 2008) and genetic diversity (Andersen et al. 2004; Angelone and Holderegger 2009; Crosby et al. 2009; Lesbarrères et al. 2002; Dixo et al. 2009). Yet, the effects of fragmentation on amphibian genetic diversity across different matrix types and different amphibian species ecologies remain to be explored.

In this study, we examined the effects of habitat fragmentation on genetic diversity in frogs of the central Brazilian Atlantic Forest (BAF). The BAF is one of the most fragmented ecosystems in the world, with only ~8% of its original forested area remaining and distributed across more than 200,000 fragments (Ribeiro et al. 2009). This fragmentation has altered community structure and diversity in amphibians (Dixo and Martins 2008) and numerous other taxa (Lôbo et al. 2011; Chiarello 1999; Maldonado-Coelho and Marini 2004; Leal et al. 2012). Despite this, the BAF remains strikingly diverse, and is home to approximately 660 described amphibian species (L. F. Toledo, *unpubl.*). We focused our study on six BAF frog species: three are considered habitat generalists based on their ability to inhabit forests as well as edge and modified habitats (Hylidae: *Boana bandeirantes, Dendropsophus minutus*, and *Dendropsophus branneri*), and three are considered habitat specialists based on their restriction to interior forest habitat (Hylidae: *Aplastodiscus leucopygius*, and *Boana semilineata*; Brachycephalidae:

Ischnocnema henselii). We collected genetic samples from these six focal species in continuous and fragmented habitats in two geographic regions: (1) southeastern São Paulo, where we compared forest fragments within an intensive cattle pasture matrix to nearby continuous preserved forest; and (2) southern Bahia, where we compared fragments within cattle pasture matrix, continuous preserved forest, and rustic vs. more managed shade cacao (cabruca) agroforests. Our study was designed to test the following hypotheses: (i) habitat fragmentation causes genetic erosion and genetic isolation in frogs; (ii) genetic erosion and genetic isolation are more severe in habitat specialists than in habitat generalists; and (iii) compared with natural forest fragments in intensive agricultural matrix (cattle pasture), rustic agriculture (cabruca) maintains higher levels of genetic diversity and population connectivity.

Methods

Study Areas

Continuous and fragmented forest habitats were sampled in two regions of the central Brazilian Atlantic Forest (Fig. 4-15): southeastern São Paulo (January-February 2016, January 2017) and southern Bahia (January-February 2017). In São Paulo, the continuous forest was a protected area of the Serra do Mar Atlantic Forest Reserve, which stretches across the states of São Paulo and Rio de Janeiro. Our sampling was conducted in the Serra do Mar headquarters known as Nucléo Santa Virginia in the vicinity of the Vargem Grande base station (23°25’S, 45°11’W, 740-1, 620 m above sea level), which is located in the municipality of São Luiz do Paraitinga. Santa Virginia is approximately 17,000 ha in total area and is dominated by typical dense Atlantic rainforest. The fragmented area in São Paulo was located in nearby in São Luiz do Paraitinga, approximately 30 km northwest of Santa Virginia. Three fragments (mean elevation

840 m asl) were sampled that had been previously surveyed for frogs by Becker et al. (2010). Fragments were selected that do not significantly differ in size and shape (C. G. Becker et al. 2010) to limit confounding effects on the amphibian community structure (Almeida-Gomes et al. 2016). The forest fragments contain Atlantic forest tree and understory plant species invaded by densely vegetated edge habitat species, and the predominant matrix between fragments is open cattle pasture.

In Bahia, frogs were sampled from continuous and fragmented forest habitats from two localities in the southern part of the state, Igrapiúna and Camacã. In Igrapiúna (hereafter referred to as the northern Bahia sampling region), continuous preserved natural forest (13° 50’ S, 39° 14’ W, 137m asl) and modified forest composed of more intensively managed cabruca cacao (cacao trees planted under a cultivated rubber tree canopy; 13° 50’ S, 39° 14’ W, 63m asl) were sampled from within the Reserva Ecológica Michelin forest reserve. The forest in the Michelin reserve has been formally protected since 2005, and covers ~1,800 hectares of lowland primary and secondary Atlantic forest, with a ~13,000 hectare forest just to the west of the preserve. The remaining ~1,200 ha of the Michelin reserve consists of wetlands and plantations including rubber tree cabruca (De Mira-Mendes et al. 2018). A single forest fragment was sampled in Igrapiúna northeast of the Michelin reserve (13° 47’ S, 39° 10’ W, 44m asl) and separated from the reserve by cattle pasture matrix. In Camacã (hereafter referred to as the southern Bahia sampling region), continuous preserved natural forest (15° 25’ S, 39° 32’ W, 185 m asl), as well as a continuous modified forest composed of rustic cabruca (cacao trees planted under natural forest canopy; 15° 25’ S, 39° 32’ W, 181 m asl) were sampled from within the Serra Bonita forest reserve. The Serra Bonita reserve is composed of ~2,300 hectares of protected primary Atlantic Forest interspersed with marginal areas of rustic cabruca cacao (Dias et al. 2014). As in

the northern Bahia sampling region, a single forest fragment was sampled in Camacã, which was located to the east of Serra Bonita (15° 25' S, 39° 27' W, 136 m asl) and separated from the preserved Serra Bonita forest by cattle pasture matrix and rural human settlements.

Study Species and Sample Collection

We selected four species from São Paulo (SP) and two from Bahia (BA) to serve as focal taxa for population genetic analyses. Three of these were habitat generalists, all of which are members of the Hylidae: *Boana bandeirantes* (SP), which breeds in smaller bodies of standing water with abundant reedy vegetation; and *Dendropsophus minutus* (SP) and *D. branneri* (BA), which breed prolifically in disturbed areas such as agricultural ponds and roadside ditches (Haddad et al. 2008). The remaining three focal species are forest habitat specialists: the hylid *Aplastodiscus leucopygius* (SP), which breeds in bromeliads; the brachycephalid *Ischnocnema henselii*, a direct-developing frog that spends its entire life cycle in forest leaf litter; and the hylid *Boana semilineata* (BA), which breeds primarily in large lentic bodies of water surrounded by forest (Haddad et al. 2008).

Visual and auditory surveys were used to locate frogs after nightfall in each study area. Frogs were hand-captured using individual clean plastic collection bags. Frogs were either non-lethally sampled with toeclipping or euthanized before liver tissue was removed (IACUC protocols for humane animal care and use PRO00005605 and PRO00007691; see Fig. 4-15 for sampling locations for each species). Euthanized frogs were deposited as vouchers in the Museum of Zoology at University of Campinas (ZUEC). Tissue samples were stored in 95% EtOH at -20C until laboratory processing.

Population Genetics Analysis

Genomic DNA was isolated from tissue samples using Qiagen DNeasy kits (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Double-digest restriction site-associated DNA sequencing (ddRAD) was performed on tissue samples to produce a reduced representation library (Peterson et al. 2012). Briefly, ~200-500 ng of DNA from each individual was digested with restriction enzymes *Eco*RI and *Mse*II and ligated to a unique 10 bp barcode and Illumina HiSeq adapters. Samples were pooled by study region (n = 80 from SP, n = 90 from BA) in equimolar quantities before each library was size-selected to 350-450 bp using a Pippin prep. Each step was followed by a cleanup using AMPure magnetic beads (1.6x) to remove small DNA fragments, and quantifications were conducted using a Qubit fluorometer assay. The two pooled libraries were each sequenced in a single lane of Illumina HiSeq2500 (150bp single-end reads) at the Centre for Applied Genomics (Hospital for Sick Children, Toronto, ON, Canada).

The resulting sequence data were demultiplexed and processed using the Stacks bioinformatics pipeline (Catchen et al. 2011, 2013). Briefly, after dividing each library dataset by frog species, sequences were demultiplexed by barcode and quality-filtered using a mean Phred score threshold of 10 (*process_radtags*), then clustered into loci (*ustacks*) within individuals with a minimum coverage (-m) of 4 reads per allele (8 reads per homozygous allele) and ≤ 3 nucleotide differences between sequences (-M). Individuals were then compared against the full catalog of species-specific loci (created with *cstacks*) within the library and genotyped at all loci (*sstacks*). Because loci exhibited variable coverage across individual samples, loci were filtered according to coverage across individuals until sequencing rates (% individuals sequenced at a given locus) were achieved of ≥ 80% for the São Paulo library and ≥ 90% for the Bahia library.

A single SNP was randomly selected from each retained locus for analysis. Individuals were genotyped and summary statistics were calculated (*populations*) to compare genetic diversity across populations (fragmented vs. continuous in SP, fragmented vs. continuous preserved vs. continuous modified in BA). The mean and standard error of the mean were calculated across all SNPs for four summary statistics that represent measures of genetic diversity: expected heterozygosity (H_E) and observed heterozygosity (H_O), nucleotide diversity (π), and inbreeding coefficient (F_{IS}). These summary statistics were statistically evaluated among populations within species in R (vrs. 3.5.1) using t-tests or one-way ANOVA tests after confirming that the data met the assumptions of parametric tests. To assess genetic structure among populations, the average and standard error of the mean fixation index (F_{ST}) was calculated method across all loci using an AMOVA and evaluated for departure from 0. F_{ST} was compared across São Paulo species using t-tests to analyze effects of species ecology (generalist vs. specialist) and across Bahia species using Kruskal-Wallis tests to analyze effects of habitat type (fragmented vs. continuous preserved vs. continuous modified) on genetic differentiation. To assess genetic isolation among populations as a proxy of dispersal, Principle Coordinate Analyses (PCAs) were conducted and visualized using the R packages ade4 (Dray and Dufour 2007) and factoextra (Kassambara et al. 2017).

Results

Impacts of landscape modification on genetic diversity

In São Paulo, genetic diversity was lower in fragmented relative to continuous populations of the generalists *D. minutus* and *B. bandeirantes* by H_E (t-tests, *D. minutus* H_E: $t(7660) = 12.2329$, $p < 0.0001$; *B. bandeirantes* H_E: $t(8468)=9.2370$, $p <0.0001$; Fig. 4-16A) but

there were no significant differences in H_O (Table 4-4). However, genetic diversity was significantly higher in fragmented populations relative to continuous populations by H_E in both specialists *A. leucopygius* and *I. henselii* (t-tests, *A. leucopygius*: $t(7794)= 2.1859$, $p <0.01$; *I. henselii*: $t(10791) = 22.4971$, $p < 0.0001$ Fig. 4-16A), and by H_O in *I. henselii* (t-test, $t(7794)=8.7180$, $p < 0.0001$; Table 4-4). Nucleotide diversity, π, was lower in fragmented relative to continuous populations in three of the four São Paulo species (t-tests, *A. leucopygius*: $t(7794) = 8.8761$, $p < 0.0001$; *D. minutus*: $t(7660) = 7.6106$, $p < 0.0001$; *B. bandeirantes*: $t(8468) = 697.664$, $p < 0.0001$), but in *I. henselii*, π was significantly higher in the fragmented population (t-test, $t(10791)=18.91$, $p < 0.0001$; Table 4-4). Inbreeding, F_{IS}, was significantly higher in the fragmented population of *I. henselii* relative to the continuous population (t-test, $t(10791) = 756.5104$, $p < 0.0001$) but F_{IS} was significantly higher in continuous populations of the three remaining species (t-tests, *A. leucopygius*: $t(7794) = 82.1582$, $p < 0.0001$; *D. minutus*: $t(7660) = 344.2218$, $p < 0.0001$; *B. bandeirantes*: $t(8468) = 351.1334$, $p < 0.0001$; Table 4-4).

The two focal species of Bahia each showed distinct patterns of genetic diversity across sampling regions and habitat types. In the generalist *D. branneri,* genetic diversity did not vary across southern populations for any of the four measures (t-tests, $p > 0.05$; Fig. 4-16B; Table 4-4). Among the northern Bahia populations, genetic diversity was significantly lower in the fragmented population relative to the northern continuous preserved population according to H_E (ANOVA, $F(4,83421) = 38.35$, $p < 0.0001$; Fig. 4-16B) but was not different among any of the three northern Bahia habitats according to H_O (Table 4-4). Nucleotide diversity was significantly different across all three northern Bahia habitats, with the highest π in the modified continuous population, and the lowest π in the fragmented population (ANOVA, $F(2, 50060) = 17.51$, $p < 0.0001$; Table 4-4). Inbreeding followed the same pattern as nucleotide diversity, with F_{IS} highest

in the continuous modified population, and lowest in the fragmented population (ANOVA, $F(4, 50060) = 10.9986$, $p < 0.0001$; Table 4-4).

In the Bahia specialist *B. semilineata*, the southern populations were statistically different for all four measures of genetic diversity. For H_E and π, genetic diversity was higher in the continuous modified population (t-tests, H_E: $t(71676) = 20.8122$, $p < 0.0001$; π: $t(71676) = 23.4881$, $p < 0.0001$; Fig. 4-16B). For H_O, genetic diversity was higher in the continuous preserved population (t-tests, $t(71676) = 3.1333$, $p < 0.01$; Table 4-4). F_{IS} was also significantly higher in the continuous modified population relative to the continuous preserved population (t-test, $t(8993) = 25.2481$, $p < 0.0001$; Table 4-4). In the northern populations of *B. semilineata*, genetic diversity did not vary between the continuous modified and fragmented population in the north, and was significantly lower in the continuous preserved population for both measures of heterozygosity (H_E: ANOVA, $F(2,107511) = 15.0655$, $p < 0.0001$, Fig. 4-16B; H_O: ANOVA, $F(2,107511) = 15.3548$, $p < 0.0001$; Table 4-4). There were no differences in nucleotide diversity or inbreeding among the northern *B. semilineata* populations.

Genetic differentiation and isolation across species and landscapes

Significant genetic differentiation was observed in all species, with nonzero within-species pairwise F_{ST} values in all population comparisons. Pairwise F_{ST} values varied according to species ecology in the São Paulo species, with the specialists *A. leucopygius* and *I. henselii* exhibiting significantly higher average F_{ST} across all loci in fragmented vs. continuous habitats relative to the generalists *D. minutus* and *B. bandeirantes* (t-test, $t(40296) = -24.66$, $p < 0.0001$; Fig. 4-16C). F_{ST} also varied among the Bahia species, with the specialist *B. semilineata*

exhibiting lower F_{ST} values on average than the generalist *D. branneri* (Mann-Whitney U test, standardized U = -37.211, $p < 0.001$; Table 4-5).

To test whether agroforests (modified continuous habitats) maintain frog dispersal, F_{ST} was compared across habitat type pairs within the northern sampling region. In both Bahia species, F_{ST} was significantly higher for fragmented-preserved habitat population pairs than for modified-preserved continuous habitat population pairs (Kruskal-Wallis test for all 10 population pairs followed by Dunn-Bonferroni post-hoc pairwise comparisons tests, *D. branneri*: 0.056 vs. 0.048, KW $H(9) = 12254.636$, $p < 0.001$, DB $p < 0.01$; *B. semilineata*: 0.054 vs. 0.049, KW $H(9) = 22175.132$, $p < 0.001$, DB $p < 0.01$; Fig. 4-16D).

In São Paulo, genetic isolation also varied according to species ecology. Specialists exhibited complete genetic isolation between fragmented and continuous habitats (Fig. 4-17A&B). Of the two generalists, *D. minutus* showed genetic overlap between fragmented and continuous habitats (Fig. 4-17D), while *B. bandeirantes* showed overall separation although there was some overlap across populations, with one individual sampled from the continuous forest clustering with the fragmented population on the PCA (Fig. 4-17E). For the two focal Bahia species, there was a clear separation between the northern and southern sampling regions (Fig. 4-17C&F) although there was one northern *D. branneri* individual that clustered with the southern continuous preserved population. In the northern sampling region, in both species the continuous modified population overlapped with the continuous preserved and fragmented populations. In the southern sampling region, distinct genetic clusters formed in both species. In *D. branneri* there was not complete separation between southern fragmented and continuous preserved habitats although the populations formed clear clusters (Fig. 4-17F). In *B. semilineata*

there was complete genetic isolation between the southern continuous preserved and modified habitats (Fig. 4-17C).

Discussion

Effects of fragmentation on genetic diversity vary by species ecology

In this study, we evaluated the genetic effects of habitat modification in the Brazilian Atlantic Forest across frog species with divergent ecologies. Our results partially support our first hypothesis that habitat fragmentation reduces genetic diversity, but surprisingly we only consistently recovered this pattern in habitat generalists. The overall patterns we observed for generalist frogs are somewhat consistent with previous findings from the high dispersing habitat generalist bufonid *Rhinella ornata* in the central Brazilian Atlantic Forest, which showed a similar loss of genetic diversity due to fragmentation (Dixo et al. 2009).

Contrary to expectations, forest specialists exhibited significantly higher genetic diversity in fragmented areas. Funk *et al.* (2005) posited that low dispersing species (such as the forest specialists in this study) experience little to no impacts of fragmentation. This could explain a lack of genetic erosion with fragmentation in forest specialists, but it does not explain genetic diversity being higher in fragmented specialist populations relative to continuous ones. A possible explanation for this seemingly paradoxical result is that contemporary fragment populations originated from multiple source populations with historically high levels of population structure. This hypothesis is supported by a previous large-scale phylogeographic study of frogs across Atlantic Forest biomes, which showed that forest specialists possess historical genetic structure that predates human deforestation in the Atlantic Forest, while generalists show little to no structure (Carnaval 2006). This also holds true across a broader

sample of tropical frogs from across the world (Rodríguez et al. 2015), indicating that this may be a common characteristic of forest specialist frogs.

It is possible that due to a combination of historical population structure and life history attributes such as relatively long generation times, there has been insufficient time for genetic erosion to occur in the fragmented specialist populations. Theoretically, at least 10-100 generations are required for genetic erosion following isolation of a population (Mona et al. 2014). An alternative explanation for the pattern we recovered is that microevolutionary pressures differ between continuous and fragmented environments: the stable environment of a continuous forest may select for similar genotypes and provide a means for forest specialists to inbreed *in situ* year to year, whereas a forest fragment environment would be subject to greater fluctuation and select for different genotypes year to year. Further data on the focal species' life history as well as long-term field surveys and genetic studies would be needed to test these alternative hypotheses.

Genetic isolation results from fragmentation in higher elevation forest specialists

We analyzed genetic structure to test our hypothesis that fragmentation more severely isolates forest specialists than habitat generalists. Our analyses of genetic differentiation (F_{ST}) and genetic isolation (PCA) aligned with our expectations in the São Paulo study region: we observed greater genetic structure and greater isolation between fragmented and continuous habitats in specialists relative to generalists, which implies that fragmentation leads to severely reduced dispersal in forest specialists. While there have been no studies on the effects of contemporary fragmentation on genetic structure and isolation in forest specialists in the Atlantic Forest, the generalist *R. ornata* was found to exhibit low genetic isolation due to fragmentation

(Dixo et al. 2009) much like our generalist focal species. Multi-species studies in other taxa have shown this relationship between habitat specialization and isolation due to fragmentation, including in mice (Mech and Hallett 2001), beetles (Brouat et al. 2003), and spiders (Vandergast et al. 2004).

In Bahia, genetic isolation was more pronounced in the specialist *B. semilineata*, with the southern populations exhibiting complete genetic isolation between modified and preserved continuous populations, and the northern populations exhibiting discrete though incompletely separated clusters in the continuous preserved vs. fragmented population. The *B. semilineata* population in the northern modified forest (cacao under rubber trees) overlapped with both the nearby preserved forest and fragment population, and F_{ST} values were relatively low though nonzero across all pairs of these habitats (0.049-0.054). For the generalist *D. branneri*, there was little evidence of complete genetic isolation in either the southern or northern Bahia sampling region. F_{ST} values were substantially lower between fragmented and continuous populations within sampling regions in Bahia relative to São Paulo (~0.055 in both Bahia species compared with an average of 0.07 in São Paulo species) suggesting that in general dispersal across habitats is higher for the Bahia species. Hypothetically this may be due to the lowland Bahia species having higher tolerances for the relatively high temperatures and low humidity found in cattle pastures compared with the mid- to high-elevation São Paulo species.

The F_{ST} values that we recovered between fragmented and continuous habitats within sampling regions are relatively low (< 0.1) compared with other studies of fragmented amphibians. For example, Lesbarrères et al. (2002) report average F_{ST} value of 0.2 in fragmented *R. temporaria* populations that had been isolated for only 20 years. This difference in results may be attributed to geographic scale contributing to greater baseline genetic structure: Lesbarrères et

al. compared populations more than 10 km apart, which may have originated from different metapopulations, while our within-region population comparisons were conducted across ≤ 10 km. However, even the F_{ST} values between Bahia regions, over a distance of ~200 km, reached only 1.5. The relatively low F_{ST} values we recovered may be due to the large number of genome-wide markers used in this study relative to traditional studies of genetic structure based on microsatellites. Alternatively, our results may reflect biased sampling of males relative to females: in opportunistic amphibian surveys, males are more likely to be located and captured because only males vocalize. Given that many frogs are matrilocal (*i.e.*, exhibit greater dispersal in males than females), males should show lower genetic differentiation than females (Henle et al. 2014). Nonetheless, we recovered non-zero F_{ST} values between all population pairs in our study, and thus were able to detect genetic structure reflecting habitat fragmentation and modification.

Impacts of rustic vs. intensive landscape modification

Our results from the northern Bahia sampling region partially support our hypothesis that continuous modified areas could maintain higher dispersal and genetic diversity relative to fragmented areas. First, across all measures, genetic diversity was equally high or higher in populations from continuous modified habitats (shade cacao) relative to continuous preserved habitat populations of both focal species across both sampling regions in Bahia. In fact, the *H. semilineata* population in the rustic cabruca agroforest in the southern Bahia sampling region had the highest genetic diversity out of all Bahia populations. While neither the generalist *D. branneri* nor the specialist *B. semilineata* exhibited genetic isolation across fragmented vs. continuous modified vs. preserved habitats, F_{ST} values were significantly higher in both species

in fragmented-preserved habitat compared with modified-preserved continuous habitat. However, in the southern region, the *B. semilineata* continuous modified population was genetically isolated from the preserved continuous population, indicating that there is not necessarily high connectivity between continuous and preserved habitats in all sampling regions. Nonetheless, our results overall support the idea that rustic forms of agriculture maintain genetic diversity and dispersal of sensitive amphibian species. This corroborates previous findings that rustic agriculture maintains higher amphibian dispersal than cattle pasture (Pineda and Halffter 2004).

Conclusion

Taken together, our results suggest that frog genetic diversity has not eroded in specialists found in these recently fragmented systems of the Brazilian Atlantic Forest. If genetic diversity is taken as a proxy for population fitness and resilience, this implies that small forest patches can support populations that are likely to survive into the future, and that these patches constitute areas of high conservation value. In previous studies, relatively high herpetofaunal diversity was maintained across a network of forest patches (Bell and Donnelly 2006). Our results also support the conservation value of rustic agricultural practices that can maintain vertebrate genetic diversity. As we detected genetic isolation in specialists, conservation efforts in this region may be best focused on improving habitat connectivity to retain frog genetic diversity in this region, such as stepping stone habitats which have proven effective at restoring migration and genetic diversity in frogs (Angelone and Holderegger 2009). In conclusion, our findings indicate that seemingly low-quality habitats such as intensively modified agroforests and small forest patches

are potentially valuable components of the broader ecosystem and should be included in conservation and management plans.

TABLES

Table 4-4: Population genetic summary statistics for ddRAD loci.

Mean and standard error (SE) values are given across all SNPs within each population. Abbreviations are as follows: Ho = observed heterozygosity, π = nucleotide diversity, Fis = inbreeding coefficient. Bolded values indicate a significant difference in t-tests (São Paulo) or ANOVA (Bahia). Values highlighted in yellow indicate that these populations (continuous modified vs. continuous preserved *B. semilineata*) were not different in pairwise Tukey's HSD post-hoc tests.

SÃO PAULO	Fragmented		Continuous		Fragmented		Continuous		Fragmented		Continuous		Fragmented		Continuous	
Species	n ind	n SNPs	n ind	n SNPs	Ho mean	Ho SE	Ho mean	Ho SE	π mean	π SE	π mean	π SE	Fis mean	Fis SE	Fis mean	Fis SE
A. leucopygius	9	4610	4	3186	0.23	0.002	0.24	0.0021	**0.26**	**0.0016**	**0.28**	**0.0019**	0.08	0.006	0.09	0.005
I. henselii	6	7948	5	2845	**0.18**	**0.0013**	**0.16**	**0.0016**	**0.24**	**0.0013**	**0.19**	**0.0016**	**0.15**	**0.0055**	**0.07**	**0.003**
D. minutus	5	2911	7	4751	0.12	0.0018	0.11	0.0015	**0.25**	**0.0023**	**0.28**	**0.0019**	**0.26**	**0.0107**	**0.36**	**0.013**
B. bandeirantes	7	3657	8	4813	0.17	0.0015	0.17	0.0014	**0.22**	**0.0015**	**0.24**	**0.0014**	**0.12**	**0.0075**	**0.17**	**0.007**

BAHIA NORTH	Fragmented		Continuous Modified		Continuous Preserved		Fragmented		Continuous Modified		Continuous Preserved		Fragmented		Continuous Modified		Continuous Preserved		Fragmented		Continuous Modified		Continuous Preserved	
Species	n ind	n SNPs	n ind	n SNPs	n ind	n SNPs	Ho mean	Ho SE	Ho mean	Ho SE	Ho mean	Ho SE	π mean	π SE	π mean	π SE	π mean	π SE	Fis mean	Fis SE	Fis mean	Fis SE	Fis mean	Fis SE
B. semilineata	8	35839	9	35839	6	35836	**0.12**	**0.0009**	**0.12**	**0.0009**	**0.12**	**0.001**	0.14	0.001	0.14	0.0009	0.14	0.001	0.05	0.0041	0.047	0.0041	0.051	0.005
D. branneri	5	16685	7	16689	9	16689	0.10	0.0014	0.10	0.0012	0.10	0.0012	**0.14**	**0.0015**	**0.15**	**0.0014**	**0.14**	**0.0014**	**0.076**	**0.0065**	**0.13**	**0.0069**	**0.11**	**0.009**

BAHIA SOUTH	Fragmented		Continuous Modified		Continuous Preserved		Fragmented		Continuous Modified		Continuous Preserved		Fragmented		Continuous Modified		Continuous Preserved		Fragmented		Continuous Modified		Continuous Preserved	
Species	n ind	n SNPs	n ind	n SNPs	n ind	n SNPs	Ho mean	Ho SE	Ho mean	Ho SE	Ho mean	Ho SE	π mean	π SE	π mean	π SE	π mean	π SE	Fis mean	Fis SE	Fis mean	Fis SE	Fis mean	Fis SE
B. semilineata	NA	NA	8	35839	12	35839	NA	NA	**0.14**	**0.0009**	**0.14**	**0.0012**	NA	NA	**0.16**	**0.0009**	**0.13**	**0.001**	NA	NA	**0.07**	**0.0052**	**-0.0162**	**0.006**
D. branneri	6	16679	NA	NA	6	16684	0.096	0.0013	NA	NA	0.097	0.0013	0.1337	0.0015	NA	NA	0.13	0.0015	0.0845	0.0085	NA	NA	0.0841	0.008

Table 4-5: Genetic differentiation (pairwise mean AMOVA F_{ST}) across all Bahia focal species populations.

B. semilineata	fragmented north	modified north	continuous north	modified south
modified north	0.043813522			
continuous north	0.054063473	0.048969797		
modified south	0.087367592	0.084036627	0.088310267	
continuous south	0.127412136	0.122330239	0.131486956	0.078092359
D. branneri	**fragmented north**	**modified north**	**continuous north**	**fragmented south**
modified north	0.063849712			
continuous north	0.056210969	0.048151977		
fragmented south	0.161162855	0.120784491	0.124385902	
continuous south	0.162548683	0.122933772	0.126762884	0.069251791

FIGURES

Figure 4-15: Map of sampling sites in São Paulo and Bahia.
White circles are continuous preserved forested areas, red triangles are small forest fragments, and orange circles are continuous modified cacao agroforests. São Paulo fragments contained subsets of the focal species: FR1 = only *D. minutus*, FR2 = *A. leucopygius* and *B. bandeirantes*, and FR3 = only *I. henselii.* Forest preserves are as follows: CO1 = Serra do Mar Nucléo Santa Virginia, CO2 = Serra Bonita, CO3 = Reserva Ecologica Michelin. Cacao agroforests are as follows: MO1 = rustic cabruca cacao, MO2 = managed cabruca cacao with rubber tree canopy. Sample sizes for individuals included in genetic analyses from each study site can be found in Table 4-4.

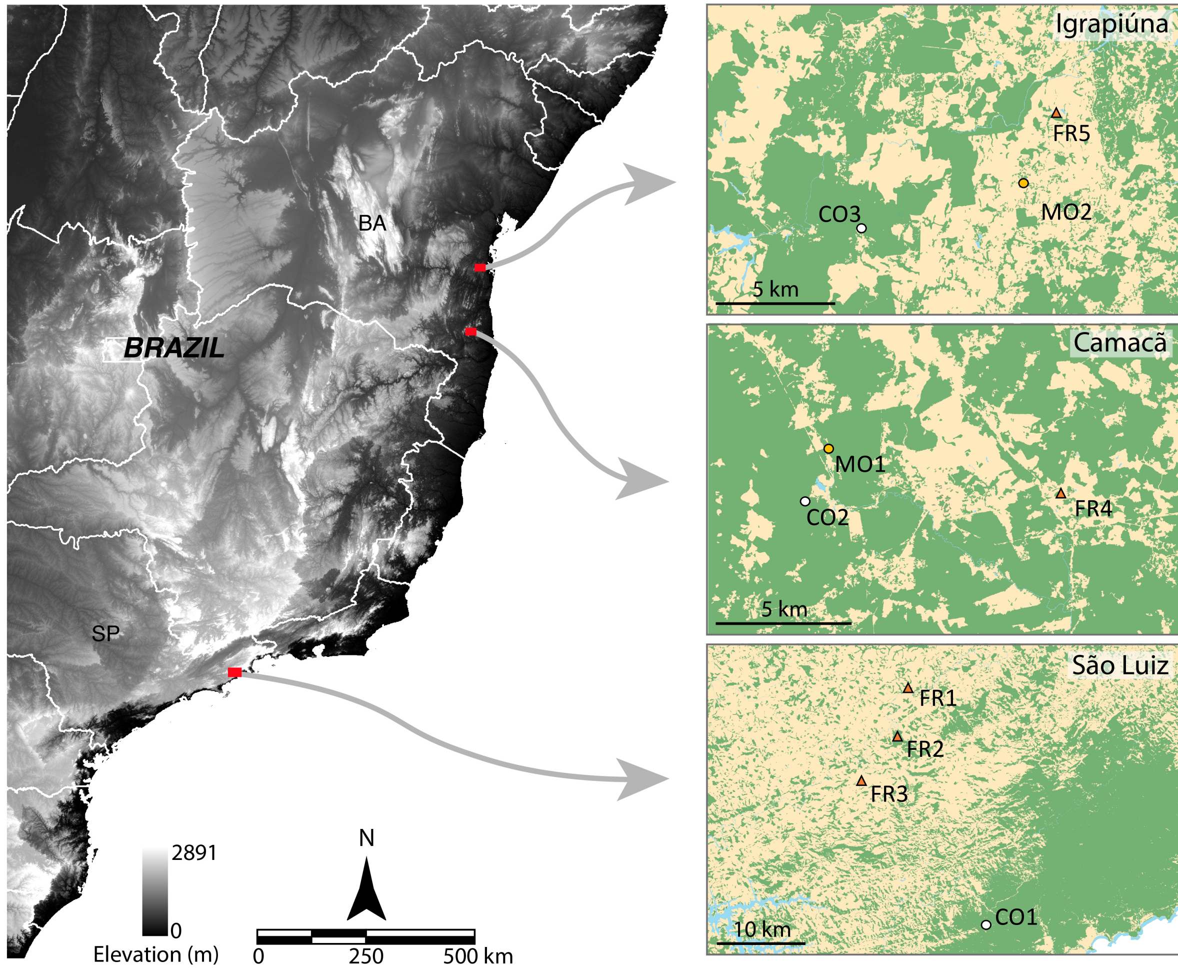


Figure 4-16: MHC IIB genetic diversity and genetic structure across the six focal frog species. São Paulo focal species are shown in A and C, and Bahia focal species are shown in B and D. Error bars represent 2 SE above and below the mean. Asterisks indicate a significant difference between bars in t-tests (alpha = 0.05). **(A)** Relative to continuous populations, heterozgosity is higher in fragmented populations of forest specialists *Aplastodiscus leucopygius* and *Ischnocnema henselii*, and lower in fragmented populations habitat generalists *Dendropsophus minutus* and *Boana bandeirantes*. **(B)** Relative to the continuous population in the northern Bahia sampling region, heterozygosity is similar in the fragmented specialist *B. semilineata* population, but is lower in the habitat generalist *D. branneri* population. Heterozygosity is similar in the modified continuous habitat (intensive/managed rubber tree cabruca) relative and continuous preserved forest in the habitat generalist *D. branneri* in the northern sampling area. Heterozygosity in continuous modified habitats exceeds heterozygosity in continuous preserved habitats in the specialist *B. semilineata* in both sampling areas. **(C)** Genetic differentiation (nonzero F_{ST}) occurs in all four São Paulo species between fragmented and continuous sites, but is higher in specialists than in generalists. **(D)** Genetic differentiation (nonzero F_{ST}) occurs across northern Bahia sampling regions in both species. Genetic differentiation is higher between fragmented and continuous preserved habitats than between continuous modified and continuous preserved habitats in both species.

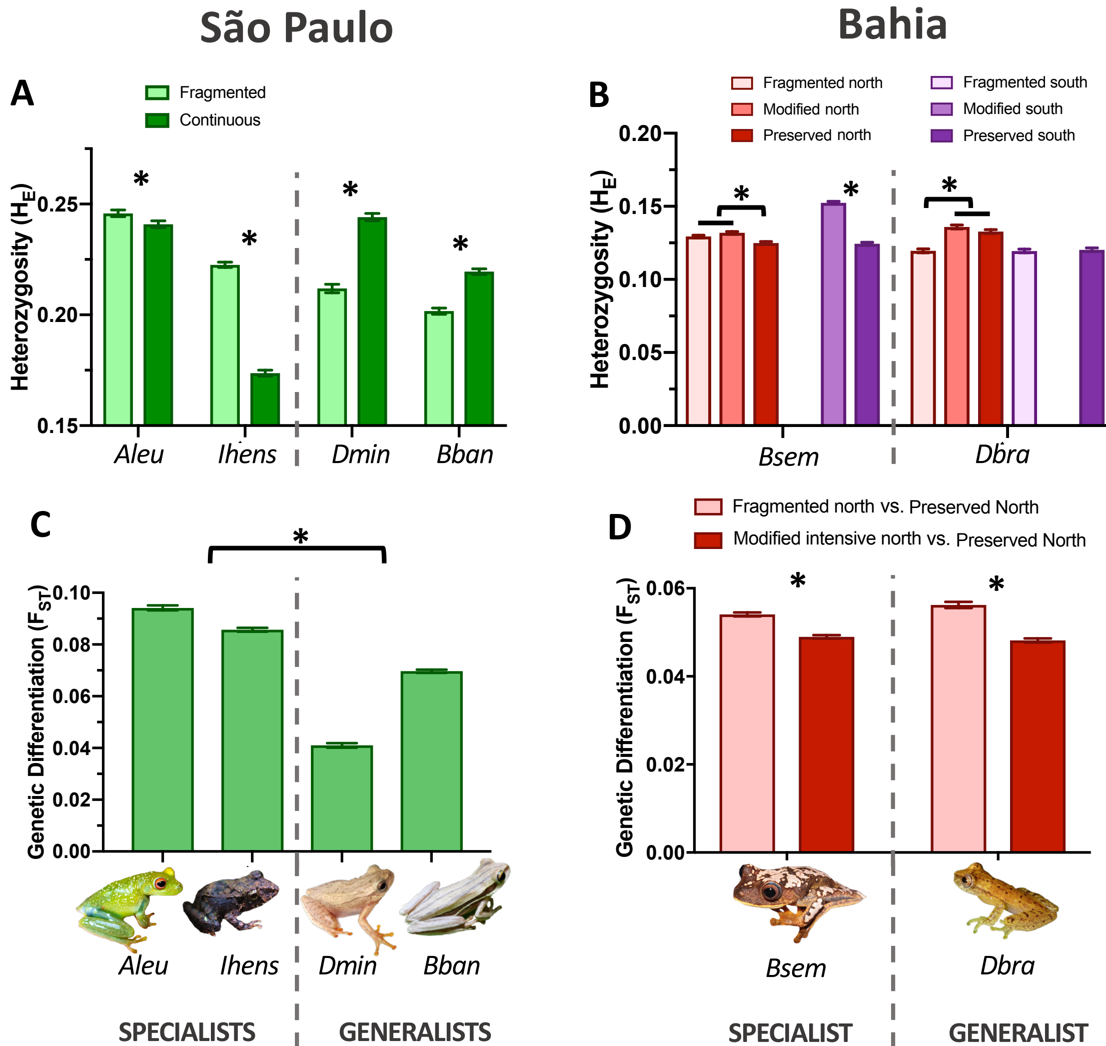
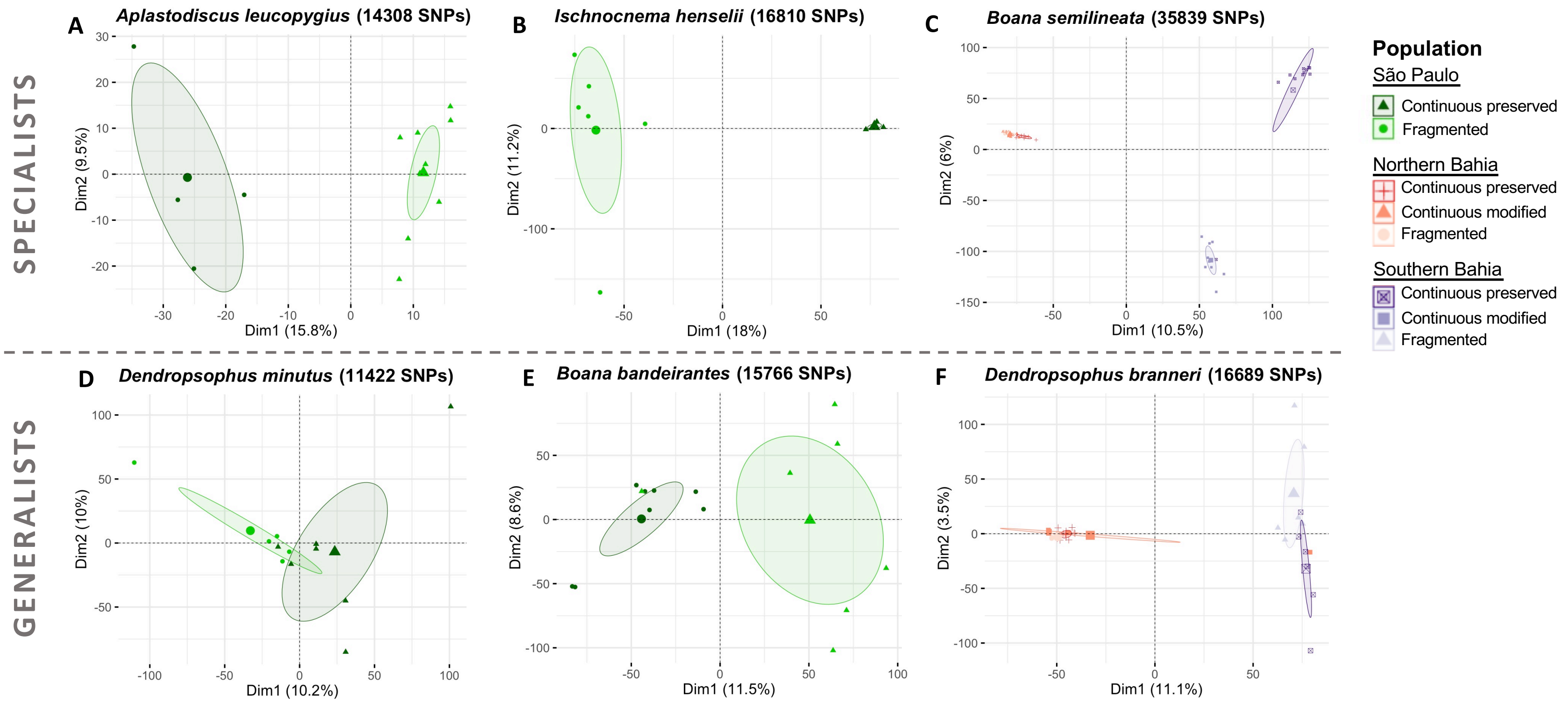


Figure 4-17: Principle Components Analysis plots for the six focal species based on ddRAD genetic markers.
For each species, the number of SNPs retained after filtering and included in the PCA is listed in parentheses. Ellipses represent population membership at 95% confidence. Overlapping ellipses indicate gene flow between populations while non-overlapping ellipses indicate genetic isolation. Results for forest specialists are shown in A-C, and results for habitat generalists are shown in D-F. Results from São Paulo species are shown in A, B, D, and E, while Bahia species are shown in C and F.



Chapter 5: Habitat Fragmentation in the Brazilian Atlantic Forest is Associated with Erosion of Frog Immunogenetic Diversity and Increased Fungal and Apicomplexan Infections[4]

Abstract

Amphibians are globally threatened by habitat fragmentation and infectious disease, but little is known about how these two threats interact. In this study, we examined the effects of habitat fragmentation in the Brazilian Atlantic Forest on frog genetic diversity at an immune locus known to affect disease susceptibility in amphibians, the MHC IIB locus. We also used molecular analyses to quantify infections by the amphibian fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) and apicomplexan blood parasites using molecular analysis of skin swab and tissue samples, respectively. We compared fragmented and continuous forest sites in two regions of the Atlantic Forest, southeast São Paulo and southern Bahia, and sampled six frog species including forest specialists and habitat generalists. Our results indicate that habitat fragmentation is associated with genetic erosion at the MHC IIB locus, and that this is more severe in forest specialists. We recovered a pattern consistent with an MHC heterozygote advantage for *Bd*, but higher MHC IIB heterozygosity was associated with higher apicomplexan infection loads across populations. Finally, we found that three MHC IIB supertypes were associated with *Bd* prevalence and apicomplexan loads. One of these supertypes was associated with decreased susceptibility to both pathogens, and the remaining were associated with a tradeoff: frogs possessing either of these supertypes exhibited increased risk of *Bd* infection but lower

[4] This chapter will be submitted for publication with the following coauthors: Kevin R. Amses, Rebecca A. Clemons, C. Guilherme Becker, Iris Holmes, L. Felipe Toledo, and Timothy Y. James.

apicomplexan loads. Our results suggest that habitat fragmentation increases infection susceptibility in amphibians, and that this is likely mediated in part through loss of diversity and changes in allelic composition at the MHC IIB immunogenetic locus.

Introduction

Amphibians are in decline worldwide due to anthropogenic stressors including habitat modification and emerging infectious disease (Stuart et al. 2007; C. G. Becker et al. 2010; Scheele et al. 2019). The recent rise in amphibian disease worldwide has raised questions about whether pathogen virulence and/or amphibian susceptibility has increased. One hypothetical mechanism for a global rise in amphibian disease susceptibility could be related to habitat modification: the negative impacts of habitat modification on amphibians may have surpassed a threshold, tipping previously stable populations to a point of extreme disease susceptibility. These negative impacts include (1) physiological stress due to altered abiotic and biotic conditions in modified habitats, which can reduce immunocompetence in amphibians (Carey et al. 1999), and (2) loss of genetic diversity in habitats modified through habitat fragmentation, which can indirectly reduce population-level fitness and resilience (Allentoft and O'Brien 2010).

Habitat modification can reduce the genetic diversity of surviving wildlife populations (Lesbarrères et al. 2002; Andersen et al. 2004; Johansson et al. 2007; Frankham et al. 2002) or impact selection for immunogenes such as those in the vertebrate Major Histocompatibility Complex (MHC) gene family that contribute to fitness and immune function (Hernandez-Gomez et al. 2019; Gonzalez-Quevedo et al. 2016; Belasen et al. 2019). MHC genes are hypothesized to exhibit heterozygote advantage because heterozygosity maximizes pathogen recognition (L Bernatchez and Landry 2003). As a result, very high individual-level MHC diversity is common,

even in cases of highly inbred, historically fragmented populations that have lost diversity across the rest of the genome (Aguilar et al. 2004). This relationship has yet to be explored in more recently fragmented populations, although hypothetically it could hold true if genetic erosion has already taken place. On the other hand, in a number of taxa MHC diversity is naturally low or MHC diversity covaries with neutral genetic diversity and thus is driven by demographic factors and drift (reviewed in Radwan et al. 2009). If inbreeding is prevalent and genetic drift is strong enough to outweigh balancing selection, this may lead to genetic erosion even at MHC loci in recently fragmented populations. This in turn may increase susceptibility to infections on both the population- and individual-level because of the loss of rare alleles and decreased heterozygosity.

The MHC gene family is composed of two classes, with Class II genes primarily involved in the response to extracellular pathogens (Bevan 1987). In particular, the MHC class IIB exon 2 is associated with conformation of the peptide-binding region 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 on amphibians have shown that MHC IIB genotype is associated with variability in 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). In a previous single-host study, we observed genetic erosion at the MHC IIB locus in a single amphibian species that had been fragmented and isolated for 12,000-20,000 years on land-bridge islands (Belasen et al. 2019). The relationship between amphibian MHC IIB diversity has yet to be explored in more recently fragmented systems, although habitat modification has been shown to alter selection dynamics on the amphibian MHC IIB (Hernández-Gómez et al. 2019). It

remains unclear whether recent fragmentation can erode MHC IIB genetic diversity through either inbreeding and genetic drift, or altered selection.

The majority of our knowledge about the relationships between habitat modification and amphibian disease susceptibility come from studies on the amphibian fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), and the hypothesis that habitat modification increases amphibian disease susceptibility has not been well-supported in these studies. In a meta-analysis, Becker and Zamudio (2011) found that *Bd* prevalence was higher in populations living in pristine (*i.e.*, 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. 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 modification have only focused on single amphibian host species that are locally abundant habitat generalists (Becker and Zamudio 2011; Puschendorf et al. 2009; Kriger et al. 2007). These generalist species may exhibit overall hardiness to environmental factors as well as to pathogens such as *Bd*, while species that are sensitive to environmental changes or those with specialist ecologies may experience increased negative effects due to habitat modification (reviewed in Harrison and Bruna 2012). Thus, it is important to consider a diversity of species and habitat types to fully understand the impacts of habitat fragmentation on disease susceptibility in diverse tropical systems.

In addition, while the majority of previous research on amphibian disease has focused on the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), amphibians are susceptible to numerous pathogens and parasites. In lowland tropical habitats, where the climate tends to be

hotter and drier than in high elevation areas, *Bd* apparently poses a lower threat to amphibians (James et al. 2015; Scheele et al. 2019; Rebollar et al. 2016). This does not mean that frogs in lowland areas are protected from all infections, but rather that other parasites are likely to be more important in these areas. As a significant proportion of fragmented areas in the Neotropics are located at low- to mid- elevations, these less well-studied parasites may pose an overlooked threat to fragmented amphibian populations. For example, apicomplexans including *Hepatozoon* spp. are relatively common blood parasites of amphibians, which become infected either through vector (ectoparasitism) or trophic (ingestion) transmission by infected blood-feeding invertebrates (Harkness et al. 2010; Cotes-Perdomo et al. 2018). Little is known about the distribution or level of threat imposed by amphibian apicomplexan parasites in the Neotropics, although our previous work showed that apicomplexan infections were prevalent and associated with MHC IIB genotype in a single amphibian host species in lowland habitats (Belasen et al. 2019). As coinfections can have large impacts on epidemic outcomes (Susi et al. 2015), single-parasite studies likely give an incomplete picture of amphibian disease susceptibility.

In this study, we examined the effects of habitat fragmentation on MHC IIB diversity and infection prevalence in frogs of the Brazilian Atlantic Forest. More than 500 years ago the Atlantic forest stretched 1.2 million km^2 across the eastern coast of South America, but has been reduced to around ~8% of this original area due to anthropogenic deforestation and fragmentation (Ribeiro et al. 2009). The Brazilian Atlantic Forest is now distributed among 1000s of small patches of isolated forest, with the majority of patches (>80%) less than 50 hectares in area. Despite this extensive fragmentation, the Brazilian Atlantic Forest remains one of the most biodiverse regions in the world, and is inhabited by 5% of vertebrate species described on Earth and 60% of all of Brazil's threatened animals. Amphibian diversity is

extremely high in the Brazilian Atlantic Forest, which hosts ~660 described species with more than half endemic to the region (L. F. Toledo, *unpubl.*). With this high level of diversity of species and ecologies, the effects of fragmentation on amphibian genetics and disease may vary widely.

We sampled six Atlantic Forest frog species that include habitat generalists and forest specialists to examine the range of effects on immunogenetics and infections in this diverse tropical amphibian fauna. These populations were previously genotyped at neutral loci using a reduced-representation library approach (ddRAD; Belasen et al. *unpubl.*). We collected tissue samples and skin swabs from our focal species in fragmented and continuous forested habitats in two sampling regions (high vs. low elevation) to quantify immunogenetic diversity using the MHC IIB locus and assess infection prevalence and load of *Bd* and apicomplexan parasites. Our study was designed to test the following research questions: (i) How is MHC IIB diversity and allelic composition affected by fragmentation in habitat generalists vs. habitat specialists? (ii) Does fragmentation increase infection susceptibility across a range of species ecologies? (iii) Do apicomplexan blood parasites pose a more significant threat than *Bd* in lower elevation habitats? (iv) Does MHC IIB diversity or genotype correlate with infection susceptibility?

Methods

Study system and sample collection

Two regions in the Brazilian Atlantic Forest were sampled for this study that contained continuous forests as well as isolated forest fragments: southeastern São Paulo state and southern Bahia state (Fig. 5-18). In São Paulo, forest fragments that are ~200 years old and located within a cattle pasture matrix were sampled in the municipality of São Luiz do Paraitinga (23°09’S

45°15’W, 840 m asl). A section of the same original forest that has been preserved within a protected area (Nucléo Santa Virginia, Serra do Mar; 23°25’S, 45°11’W, 620 m above sea level, ~17,000 ha total area of natural forest) was sampled ~30 km from the fragmented area. Similarly, ~200 year old forest fragments within a matrix of cattle pasture were sampled in the municipality of Igrapiúna (13° 50' S, 39° 13' W, 237m asl) in southern Bahia. A continuous forested site was sampled within a nearby protected area, the Reserva Ecologica Michelin (13° 50' S, 39° 14' W, 137m asl, ~1,800 ha total area of natural forest).

Six focal species were sampled from fragmented and continuous habitats from the two regions, including both habitat specialists (those that live and reproduce only in forested areas) and generalists (those that can live and reproduce in a variety of habitats including low-quality agricultural matrix). In São Paulo, this included two habitat specialists (Hylidae: *Aplastodiscus leucopygius* and Brachycephalidae: *Ischnocnema henselii*) and two habitat generalists (Hylidae: *Dendropsophus minutus* and *Boana bandeirantes*). In Bahia this included one habitat specialist (Hylidae: *Boana semilineata*) and one habitat generalist (Hylidae: *Dendropsophus branneri*).

Frogs were individually captured at night using sterile plastic bags and were transported back to a central field laboratory for sampling. Ventral skin swab samples were taken according to a standard pathogen sampling protocol (Hyatt et al. 2007) and either lethal (liver, post-euthanasia) or non-lethal (toe) tissue samples were taken 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 Museum of Zoology at the University of Campinas in São Paulo (ZUEC) while non-lethally sampled frogs were released at the same site they were captured. DNA was extracted using a Qiagen DNeasy kit (Valencia, CA,

USA) using a modified protocol for swab samples and the standard manufacturer's protocol for tissues.

MHC IIB amplification and analysis

To analyze the MHC IIB immunogenetic locus, frog MHC primers were used to amplify and sequence a 200-400bp fragment of MHC IIB Exon 2. Tissue DNA extracts were amplified with amphibian MHC IIB primers BCF6 and BobomSR (May and Beebee 2009) and PCR products were cloned and sequenced using a TOPO TA cloning kit and blue/white screening. Successful clones were sequenced and compared against the NCBI Genbank database using blastx to confirm homoloy to MHC IIB Exon 2.

Species-specific primers were developed for two species (*D. minutus* and *A. leucopygius*) for which clean sequences could not be consistently produced using BCF6 and BobomSR, likely due to spurious amplification of paralogs. Primers were designed using a genome walking approach (Clontech Universal Genome Walker Kit 2.0) to amplify the exon and a portion of flanking intronic region to ensure only orthologous regions would be amplified. Briefly, species-specific nested MHC IIB Exon 2 primers were designed using a BCF6/BobomSR clone sequence from each species. Tissue extracts were digested with four sets of restriction enzymes, then adapters were ligated to cut ends of DNA strands. Two rounds of PCR were conducted with nested gene specific primers and nested adapter primers to amplify DNA fragments overlapping with the MHC IIB exon 2 locus. Final PCR products were then cloned and Sanger sequenced to retrieve DNA sequences containing MHC IIB Exon 2 and flanking intronic regions. These sequences were then used to design species-specific primers that would produce orthologous amplicons.

Either BCF6/BobomSR or species-specific primers were modified with an attached indexing primer overhang (Table 5-6). These then were used to PCR-amplify a ~200-400bp fragment of the MHCIIB Exon 2 from each DNA extract. After visualizing products on a 1% agarose gel to confirm amplification, PCR products were diluted and reduced-cycle PCR was used to anneal each product to Nextera oligos that contained Illumina flow cell adapters as well as a unique 10bp index on each side. The resulting dual-indexed products were visualized on a 1% agarose gel before being quantified on a Qubit fluorometer. Samples were then pooled using equimolar volumes and purified using 1.8x AMPure magnetic beads. The pooled and purified library was sequenced on the Illumina MiSeq platform (250bp paired-end nano run) at the University of Michigan Microbial Systems Molecular Biology Laboratory.

Sequences were bioinformatically processed using the Mothur MiSeq pipeline. Briefly, MiSeq output data were split by frog species before paired reads were assembled, quality-filtered to remove short sequences and ambiguous bases, aligned to a reference alignment of MHC IIB Exon 2 sequences from four frog species (downloaded from GenBank), and clustered into >99% identical "OTUs" (operational taxonomic units) that represent MHC IIB Exon 2 alleles. A threshold of 100 reads within a single individual was used to assign alleles to individual frogs. The most abundant sequence for a given OTU was extracted as the allele sequence. Individuals with >2 alleles recovered (n = 12/114) were filtered out of the dataset, as all target species are assumed to be diploid and a single orthologous locus was being targeted. A neighbor-joining phylogenetic tree was constructed using the BioNJ algorithm and HKY model in Seaview (vrs. 4.5.4; Gascuel 1997; Gouy et al. 2010) to confirm that the final set of haplotypes were orthologous. To determine whether MHC haplotypes were genetically clustered according to species relatedness or local habitat, a haplotype network was constructed and visualized using

the pegas package in R (vrs. 3.5.1; R Team 2018; Paradis 2010) across four of the focal species: *D. minutus* and *B. bandeirantes* from São Paulo, and their congeners *D. branneri* and *B. semilineata* from Bahia.

To determine the impacts of fragmentation on MHC IIB diversity, allelic diversity (N_A), observed and expected heterozygosity (H_O and H_E), and nucleotide diversity (π) were calculated in DnaSP (Librado and Rozas 2009). Mean H_E and mean π across individuals within populations were analyzed across habitat types using t-tests in R after confirming that data conformed to the assumptions of parametric statistics. MHC IIB genetic structure was evaluated among fragmented and continuous populations within each species by calculating the fixation index (F_{ST}) in R. To compare MHC IIB diversity to neutral genetic diversity, MHC IIB diversity summary statistics H_O, H_E, and π were treated as dependent variables in separate General Linear Models that included analogous summary statistics from a reduced representation DNA sequencing (ddRAD) library constructed from the same samples (Belasen et al., *unpubl*) as a fixed effect independent variable. Additional models that included habitat type and species ecology as factors were using a stepwise additive model building procedure, and adjusted R^2 values were used to select the best model for each summary statistic.

To test for signatures of selection on the MHC IIB, the ratio of non-synonymous to synonymous sites (dN/dS) was calculated for each population and the difference between dN and dS was statistically analyzed using z-tests in MEGA (vrs. 7.0.26-mac). To further examine whether neutral processes or selection were more likely to be influencing MHC IIB, MHC IIB F_{ST} was compared with mean and 95% CI AMOVA F_{ST} values from the ddRAD dataset (Spurgin and Richardson 2010).

Allele sequences were translated into amino acid sequences in MEGA (vrs. 7.0.26-mac). Sequences were aligned with a previously published frog MHC IIB dataset (Bataille et al. 2015) to identify residues hypothetically associated with antigen-binding site pocket conformation based on analogous positions in human MHC class II alleles (antigen-binding groove pockets 4, 6, 7, and 9; Bataille et al. 2015; Mulder et al. 2017; Brown et al. 1993; Tong et al. 2006).

Positively selected sites (PSS) in the amino acid alignment were identified using a fixed effects likelihood model of site selection (Weaver et al. 2018, Pond and Frost 2005). Alleles were then clustered into functional "supertypes" based on PSS amino acid physicochemical properties (z1-z5; Sandberg et al. 1998) using a BIC-based k-means clustering algorithm and discriminant analysis of principle components (DAPC) implemented in the R package adegenet (Jombart et al. 2010).

Detection and analysis of Bd and apicomplexan infections

Swabs were analyzed using a standard qPCR assay for *Bd* detection (Boyle et al. 2004). Standard curves were produced using serial dilutions (10^6-10^0 zoospore equivalents, hereafter ZE) of BAF 2, a *Bd*-GPL culture isolated from a Brazilian Atlantic forest tadpole. Samples were run in duplicate to ensure accurate quantification, and only those containing $\geq$ 1 ZE were considered positive for *Bd.*

Previously produced ddRAD data from tissue extracts (Belasen et al., *unpubl.*) were used to determine the presence of Apicomplexan parasite OTUs using custom Linux and python batch scripts. Briefly, sequences were truncated at 100bp, clustered into OTUs at 90% similarity, and compared against the NCBI Genbank database using the megablast search algorithm. Within those OTU clusters that had any matches in the database, the top $\leq$20 matches falling below the

default minimum E-value of $1x10^{-10}$ were retained. For OTUs with at least one match to Apicomplexa in the top retained hits, the OTU was assigned to Apicomplexa. This liberal taxonomic assignment strategy was used because it is far more likely that parasite sequences would inadvertently be assigned to host organism taxonomy rather than vice versa (Zhang et al. 1997). Within each sample, the proportion of sequences that were identified as Apicomplexa relative to the total number of sequences that matched any sequence in the database was calculated as a proxy for apicomplexan load.

Bd infection rates were compared across species ecologies (habitat generalist vs. forest specialist) and habitat types (fragmented vs. continuous forest) using chi-square tests. *Bd* loads were compared across species ecologies and habitats using a two-way ANOVA in R after confirming that the data conformed to the assumptions of linear models. To test whether apicomplexan parasites pose a greater threat in low elevation Bahia compared with São Paulo, apicomplexan loads were compared across regions using a Kruskal-Wallis test.

To examine the relationship between MHC IIB diversity and infections, General Linear Models were constructed with *Bd* or apicomplexan load as the dependent variable and additive stepwise combinations of the explanatory variables of MHC IIB diversity, species identity, species ecology, and habitat type. Adjusted R^2 values were compared to select the best model for each infection type. Chi-squared tests were used to compare *Bd* infection status across MHC IIB supertypes, and General Linear Models were used to determine associations between supertype and *Bd* or Apicomplexan load.

Results

Immunogenetic diversity

Across the six focal species, 72 unique haplotypes were recovered and predominantly clustered by species on the neighbor-joining tree (Fig. 5-S24). Construction of a haplotype network between congeneric species from São Paulo and Bahia showed that haplotypes tend to cluster by genus rather than by sampling area or habitat type (fragmented vs. continuous; Fig. 5-19). While most haplotypes clustered within genera, one haplotype was shared between *D. branneri* and *B. semilineata* (haplotype XL), and a second *D. branneri*-specific haplotype (haplotype XLI) clustered within *Boana* spp. haplotypes on the network.

Five codon positions across the MHC IIB alignment were found to be under strong positive selection ($dN/dS > 10$) and aligned with putative pocket residues of the peptide-binding region (Fig. 5-S25). When amino acid physicochemical properties from these five codon positions were evaluated, the 72 haplotypes condensed into seven unique MHC IIB supertypes that tended to cluster on the tree (Fig. 5-S24). Two supertypes were found only in a single species: ST1 was found only in *D. branneri* and ST6 was found only in *D. minutus*.

MHC IIB diversity was significantly lower in fragmented populations relative to continuous populations according to expected heterozygosity (H_E) in three of four São Paulo species and both Bahia species (t-tests, $p < 0.05$ for all species except *B. bandeirantes*, Fig. 5-20A; see Table 5-7 for summary data and Table 5-8 for test statistics). MHC IIB nucleotide diversity (π) was also lower in all three specialist species and in the generalist *D. branneri*, while π was significantly higher in the fragmented population of the generalist *B. bandeirantes* relative to the continuous population (t-tests, $p<0.05$ for all species except *D. minutus*; see Table 5-7 for

summary data and Table 5-8 for test statistics). For both H_E and π, the largest declines in genetic diversity were observed in the specialists *A. leucopygius* and *I. henselii* (Table 5-7).

According to dN/dS, significant signatures of positive selection were found only in the São Paulo specialists *A. leucopygius* and *I. henselii* (Table 5-7). No populations showed significant signatures of population bottlenecks according to Tajima's D (Table 5-7).

Relative to genetic differentiation (F_{ST}) across ddRAD loci, MHC IIB showed greater genetic differentiation in three species (*A. leucopygius*, *B. bandeirantes*, and *D. branneri*) and lower genetic differentiation in the remaining three species (*I. henselii*, *D. minutus*, and *B. semilineata*; Fig. 5-20C). When MHC IIB diversity summary statistics H_E, H_O, and π were compared with summary statistics generated from ddRAD data from the same populations, only MHC IIB H_O was significantly associated with ddRAD H_O, with a negative relationship across all species and populations (SLR, $\beta = -3.2$, $p < 0.05$, $R^2 = 0.37$; Fig. 5-21A).

Incidence of Bd and apicomplexan infections

Bd infections were detected in all sites sampled in São Paulo. After running a subset of samples (~50) collected from the lowland sampling area in Bahia we found ~5% prevalence of *Bd* with positive samples showing low loads (~1 ZE). As this is consistent with other findings of very low prevalence and infections of *Bd* from lowland areas in the Atlantic Forest (Lambertini et al., *unpubl*) we considered *Bd* to be functionally absent from the Bahia populations.

Within São Paulo, fragmented populations had significantly higher *Bd* infection rates relative to continuous populations ($X^2(1) = 10.783$, $p < 0.01$) and specialists showed a trend of higher *Bd* infection rates relative to generalists although this was not statistically significant ($X^2(1) = 2.458$, $p = 0.1$; Fig. 5-22A). *Bd* infection loads tended to be higher in fragmented populations and in specialists in both habitat types, although these trends were also statistically

non-significant (two-way ANOVA, $p > 0.05$; Fig. 5-22B). While *Bd* load increased with fragmentation in specialists, loads were similar across site types in generalists.

ddRAD datasets from all samples contained DNA sequences from Apicomplexans. The majority of these sequences (89%) matched GenBank OTUs classified within the family Plasmodiidae (Fig. 5-S26). Apicomplexan parasite loads did not vary according to species ecology, but were significantly higher in Bahia than São Paulo (General Linear Model, $p < 0.01$ for region, $p > 0.05$ for site type and species ecology, $R^2 = 0.10$; Fig. 5-22C).

When infections were analyzed against MHC IIB diversity, within São Paulo there was a significant negative relationship between *Bd* prevalence and population-level MHC IIB diversity for both measures of heterozygosity, and the best models included habitat type (fragmented vs. continuous) as an explanatory variable (MHC IIB H_E: $\beta = -84.61$, $p = 0.0311$, overall model $p = 0.016$, $R^2 = 0.8752$; MHC IIB H_O: $\beta = -52.64$, $p = 0.0307$, overall model $p = 0.026$, $R^2 = 0.8395$; Fig. 5-21B). In contrast, apicomplexan load was positively associated with MHC IIB diversity by H_E across all populations, and the best model included habitat type as a factor (General Linear Model, beta = 0.911 for H_E, $p = 0.01$, overall model $p = 0.03$, $R^2 = 0.54$; Tukey's HSD $p < 0.05$ for fragmented vs. continuous habitats, Fig. 5-21C). On the individual level, MHC IIB heterozygotes were significantly less likely to be infected with *Bd* ($X^2 (1) = 9.5825$, $p < 0.01$). There was no relationship between individual-level heterozygosity and *Bd* load or Apicomplexan load (t-tests, $p > 0.05$).

Of the five supertypes that occurred across multiple species (excluding species-specific supertypes ST1 and ST6), ST2, ST4, and ST5 were significantly associated with *Bd* infection status and apicomplexan loads. Frogs possessing ST2 exhibited higher incidence of *Bd* infections ($X^2 (2) = 25.203$, $p < 0.0001$) and higher Apicomplexan loads (General Linear Model, p <

0.0001, $R^2 = 0.26$), while frogs possessing ST4 or ST5 exhibited higher incidence of *Bd* infections (ST4: $X^2 (2) = 7.0872$, $p = 0.07$; ST5: $X^2 (2) = 6.1613$, $p < 0.05$) and lower Apicomplexan loads (General Linear Model, $p < 0.0001$ for ST4 and for ST5, $R^2 = 0.26$; Fig. 5-23A). *Bd* load did not vary across supertypes (General Linear Model, $p > 0.05$). Supertype heterozygotes were significantly less likely to be infected with *Bd* ($X^2 (1) = 9.1077$, $p < 0.01$), but there was no effect of supertype heterozygosity on apicomplexan load (t-test, $p > 0.05$).

Discussion

Habitat fragmentation is associated with erosion of immunogenetic diversity

In this study, we quantified the effects of landscape modification on amphibian immunogenetic diversity and infection susceptibility across host species ecologies and landscape contexts. Overall, we found that habitat fragmentation was associated with reduced immunogenetic diversity, with the most severe reductions in MHC IIB diversity in the forest specialists *A. leucopygius* and *I. henselii*. We also found that across all species, MHC IIB diversity was inversely related to overall diversity based on genome-wide markers from a previously produced ddRAD dataset. Combined with the low Tajima's D values we recovered as well as MHC IIB F_{ST} values occurring outside of 95% confidence intervals of ddRAD marker F_{ST} values within each species, this suggests that the loss of MHC IIB diversity may not exclusively be due to genetic drift or inbreeding. In half of our focal species, MHC IIB genetic differentiation was significantly lower than expected based on genome-wide genetic differentiation, suggesting that selection is maintaining similar MHC IIB alleles in different populations. This is corroborated by the MHC IIB haplotype network, which does not show clustering according to population.

Transspecific polymorphism is thought to be common at MHC genes (Klein 1987). However, among the 72 MHC IIB haplotypes we recovered, we recovered only one common haplotype between focal species. This haplotype was found in both species from Bahia, which implies that the local environment and/or local pathogens could be driving selection for this allele. At the supertype level, however, there was evidence of transspecific polymorphism, with 5/7 supertypes shared among two or more focal species.

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 pathogen recognition (Bataille et al. 2015; Mulder et al. 2017). However, the diversity of haplotypes and supertypes that we recovered are relatively lower than might be expected based on previous studies. For example, Savage et al. (2016) recovered 84 alleles and 4 supertypes across 8 populations of a single species (128 individuals). In our study, we sampled a similar number of individuals (n=114), but included six focal species spanning two families and four genera. With this level of species diversity it is somewhat surprising that only seven functional MHC supertypes were recovered. As previous studies that have identified MHC IIB supertypes in amphibians have focused on a single focal species, it is unknown how many supertypes exist across diverse amphibian species. It is possible that supertypes show a high degree of transspecific polymorphism if amphibians are subject to similar pathogens or other selective pressures. Further comparative studies of the amphibian MHC IIB are needed to test this hypothesis.

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

As we predicted, *Bd* prevalence and loads were extremely low in the lowland Bahia sampling region. However, *Bd* was present in all São Paulo populations, with the highest *Bd* prevalence and loads in fragmented populations and in forest specialist host species. On the population-level we observed an inverse relationship between MHC diversity and *Bd* prevalence and load, and on the individual-level MHC IIB heterozygotes were less likely to be infected with *Bd*. This corroborates a previous study in which MHC IIB heterozygotes were found to have lower *Bd* susceptibility (Savage and Zamudio 2011, 2016). However, another study found increased *Bd* risk in populations with higher heterozygosity, and attributed this pattern to correlations between heterozygosity, dispersal, and *Bd* transmission in populations in more diverse populations (Addis et al. 2015). In our study area, we did not observe evidence of reduced *Bd* transmission due to reduced intraspecific dispersal with habitat fragmentation. One possible explanation for this result is that generalist species transmit *Bd* across the matrix from continuous habitats to isolated forest specialist populations. As habitat generalists show moderate prevalence and relatively low *Bd* loads overall, they could be hypothetically serving as tolerant "disease vectors" within this system.

Apicomplexan loads were higher in Bahia than in São Paulo, implying that this pathogen is more important in areas that are less suitable for *Bd*. This pattern may have arisen as a result of release from competition with *Bd*, perhaps mediated through indirect immune-system mediated effects (Graham 2008). Similar to *Bd*, apicomplexan loads were higher in fragmented populations relative to continuous preserved forest populations. However, in contrast with *Bd*, apicomplexan parasites exhibited a positive association with MHC IIB diversity. This suggests that apicomplexan transmission could be more dependent on intraspecific host dispersal. Indeed,

apicomplexan parasites have been shown to exhibit host-specificity in amphibians (Kim et al. 2006). Unfortunately, we were unable to identify Apicomplexans to species or strain with our dataset, but host-specificity remains a possible explanation for the pattern we observed.

MHC IIB supertypes 2, 4, and 5 showed significant associations with *Bd* infections and Apicomplexan loads. While all three supertypes were associated with increased *Bd* infection rates, ST4 and ST5 were associated with decreased Apicomplexan loads. Therefore, it is possible that ST4 and ST5 exhibit a tradeoff between higher *Bd* susceptibility and lower apicomplexan susceptibility. In Bahia, where apicomplexan loads were higher on average, ST2 was relatively common while ST4 and ST5 were relatively rare (Fig. 5-23B). This supports the hypothesis that rare alleles provide an advantage against common pathogens. Although no supertypes were associated with protection against *Bd*, supertype heterozygotes exhibited lower *Bd* infection risk. Previous studies did not find an MHC IIB supertype heterozygote advantage against *Bd* despite heterozygote advantage at the haplotype level (Savage and Zamudio 2016). This may be due to higher concordance in functional complementarity between allelic heterozygotes and supertype heterozygotes in our focal species, or due to a larger sample size of supertypes in this study compared with previous studies.

Somewhat surprisingly, the prevalence of apicomplexan infections across our study populations was 100%. Although Hemogregarinids and Hepatozoids are more commonly reported from amphibians (Kim et al. 2006; Desser et al. 1995), 89% of Apicomplexans we recovered matched OTUs classified within the family Plasmodiidae when compared against GenBank. A caveat of our method of diagnosing infections is that the presence of apicomplexan DNA in an amphibian host does not necessarily equate to disease. Although all apicomplexans are considered parasitic, they may also inhabit amphibians as commensals. However, when

amphibians become immunocompromised, apicomplexans present in the organism can proliferate to clinical levels and become a health concern (Whitaker 2017). For this reason, we argue that apicomplexan load can be used as a proxy of relative health across individuals and populations.

In addition, as our study is correlative, it is not possible to discern whether the associations we observed between infections and the MHC IIB locus are attributed to causal relationships between immune function and infection load, or to both factors being independently associated with fragmentation. For example, MHC IIB selection dynamics may be related to agricultural factors (Hernández-Gómez et al. 2019) rather than parasites in the fragmented landscape. Likewise, parasite loads may be increased by fragmentation due to physiological stress rather than impacts on genetic diversity. The mechanistic relationship between the MHC IIB locus and *Bd* susceptibility has been demonstrated through experimental studies (Savage and Zamudio 2011), supporting a mechanistic link between immunogenotype and *Bd* risk. Although little is known about the relationship between the amphibian MHC IIB and susceptibility to plasmodiid Apicomplexans, research on humans has shown strong coevolutionary relationships between a human HLA (MHC analog) locus and the plasmodiid parasites responsible for malaria (*Plasmodium* spp.; Hill 1998). Further research is needed to confirm a similar relationship in amphibians.

Taken together, our results suggest that habitat fragmentation is associated with increased infections and decreased immunogenetic diversity in amphibians. Immunogenetic diversity may have eroded in fragmented populations through inbreeding and genetic drift, although it is likely experiencing selection as well according to our analyses of F_{ST}. Second, we have shown that fragmentation does not reduce pathogen transmission in the case of *Bd*, a generalist pathogen,

which has access to high-dispersing habitat generalist hosts that may be functionally serving as disease vectors in this system. Lastly, disease agents other than *Bd* including Apicomplexans are likely important stressors in amphibian populations in lowland areas that experience a large proportion of habitat modification. Future studies of amphibian disease should examine multiple disease agents to gain a more complete picture of susceptibility.

Tables

Table 5-6: Custom sequencing primers used to amplify the MHC IIB Exon 2 from the six focal species.

Primer name	**Overhang** + Gene specific primer sequence
BCF6_OH	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CATTGTACAATCAGGAGGAG
BobomSR_OH	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CCATAGTTGTGTTTACAGACTGTTTCCAC
Dmin_MHCIIB_F_OH	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**TGGATTACTTTGCTGCATGG
Dmin_MHCIIB_R_OH	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CCAGGGTCTCACCTTTTCTTC
Aleu_MHCIIB_F_OH	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**ACTCAGCACGTGCGGTTACT
Aleu_MHCIIB_R_OH	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGGGCAGCCATTTCTAGTTA

Table 5-7: Population-level sample sizes and summary statistics for MHC IIB and ddRAD loci.
Abbreviations are as follows: Ecology: Spec. = forest specialist, Gen. = forest generalist; Pop = population, Frag. = fragmented forest, Cont. = continuous forest; n = number of individuals that were genotyped for MHC IIB; H_O = observed heterozygosity; H_E = expected heterozygosity; SD = standard deviation; N_A = allelic richness; dN/dS = the ratio of non-synonymous to synonymous changes; z-stat is the test statistic from the dN-dS z-test (significant results with $p < 0.05$ are in bold); π = nucleotide diversity; D = Tajima's D; F_{ST} = fixation index; n loci = the number of ddRAD loci sequenced from each population. Site codes correspond to sites in Fig. 5-18.

					MHC IIB data											**ddRAD data**					
Species	**Ecology**	**Region**	**Pop**	**Site code**	**n**	**H_O**	**H_E**	**SD H_E**	**N_A**	**dN/dS**	**dN-dS z-stat**	**π**	**SD π**	**D**	F_{ST}	**n loci**	**H_O**	**H_E**	**π**	F_{ST}	F_{ST} 95% CI
Aplastodiscus leucopygius	Spec.	SP	Frag.	FR2	10	0.20	0.70	0.11	7	2.24	**1.80**	0.06	0.012	-0.67	0.152	4610	0.23	0.25	0.26	0.094	0.0019
			Cont.	CO1	7	0.43	0.89	0.06	8	1.92	**2.15**	0.09	0.009	1.16		3186	0.24	0.24	0.28		
Ischnocnema henselii	Spec.	SP	Frag.	FR3	7	0.14	0.39	0.15	3	0.85	**2.24**	0.03	0.011	-0.55	0.020	7948	0.18	0.22	0.24	0.086	0.0015
			Cont.	CO1	10	0.60	0.85	0.06	8	2.18	**2.34**	0.07	0.009	0.50		2845	0.16	0.17	0.19		
Dendropsophus minutus	Gen.	SP	Frag.	FR1	10	0.60	0.72	0.09	6	0.35	-0.88	0.01	0.002	-1.44	0.027	2911	0.12	0.21	0.25	0.041	0.0017
			Cont.	CO1	10	0.60	0.83	0.06	8	0.39	-0.89	0.01	0.002	-0.92		4751	0.11	0.24	0.28		
Boana bandeirantes	Gen.	SP	Frag.	FR2	8	0.75	0.89	0.05	8	0.94	-0.12	0.09	0.007	0.27	0.110	3657	0.17	0.20	0.22	0.070	0.0012
			Cont.	CO1	7	0.86	0.88	0.08	9	2.14	1.98	0.08	0.009	0.21		4813	0.17	0.22	0.24		
Boana semilineata	Spec.	BA	Frag.	FR4	6	1.00	0.82	0.08	10	1.67	1.24	0.04	0.004	1.16	-0.0049	35839	0.12	0.13	0.14		
			Cont.	CO2	8	0.75	0.93	0.04	6	2.04	1.48	0.06	0.003	0.64		35836	0.12	0.13	0.14	0.054	0.0009
Dendropsophus branneri	Gen.	BA	Frag.	FR4	8	0.63	0.89	0.05	8	1.36	0.99	0.06	0.006	0.58	0.064	16685	0.10	0.12	0.14		
			Cont.	CO2	9	0.78	0.95	0.03	12	0.94	-0.12	0.09	0.008	-0.04		16689	0.10	0.13	0.14	0.056	0.0014

Table 5-8: Test statistics for comparisons of MHC IIB genetic diversity (HE and π) across populations. Independent samples t-test test statistics are report along with degrees of freedom in parentheses as well as corresponding p-values. Statistics in bold represent a significant result. All comparisons were among fragmented and continuous populations of each species.

Species	H_E **t-test statistic (df)**	H_E **p value**	**π t-test statistic (df)**	**π p value**
A. leucopygius	**4.34 (15)**	**< 0.0001**	**4.7802 (15)**	**< 0.001**
I. henselii	**8.9512 (15)**	**< 0.0001**	**9.1397 (15)**	**< 0.001**
D. minutus	**3.2738 (18)**	**< 0.01**	0.3859 (18)	> 0.05
B. bandeirantes	0.3913 (13)	> 0.05	**2.3287 (13)**	**< 0.05**
B. semilineata	**3.4215 (12)**	**< 0.01**	**7.0792 (12)**	**< 0.0001**
D. branneri	**2.832 (15)**	**< 0.05**	**10.1281 (15)**	**< 0.0001**

Figures

Figure 5-18: Map of sampling locations.
Preserved continuous forests (CO1 and CO2) are denoted with white circles, and forest fragments (FR1-FR4) are denoted with red triangles. See Table 5-7 for sample sizes and species associated with each site.

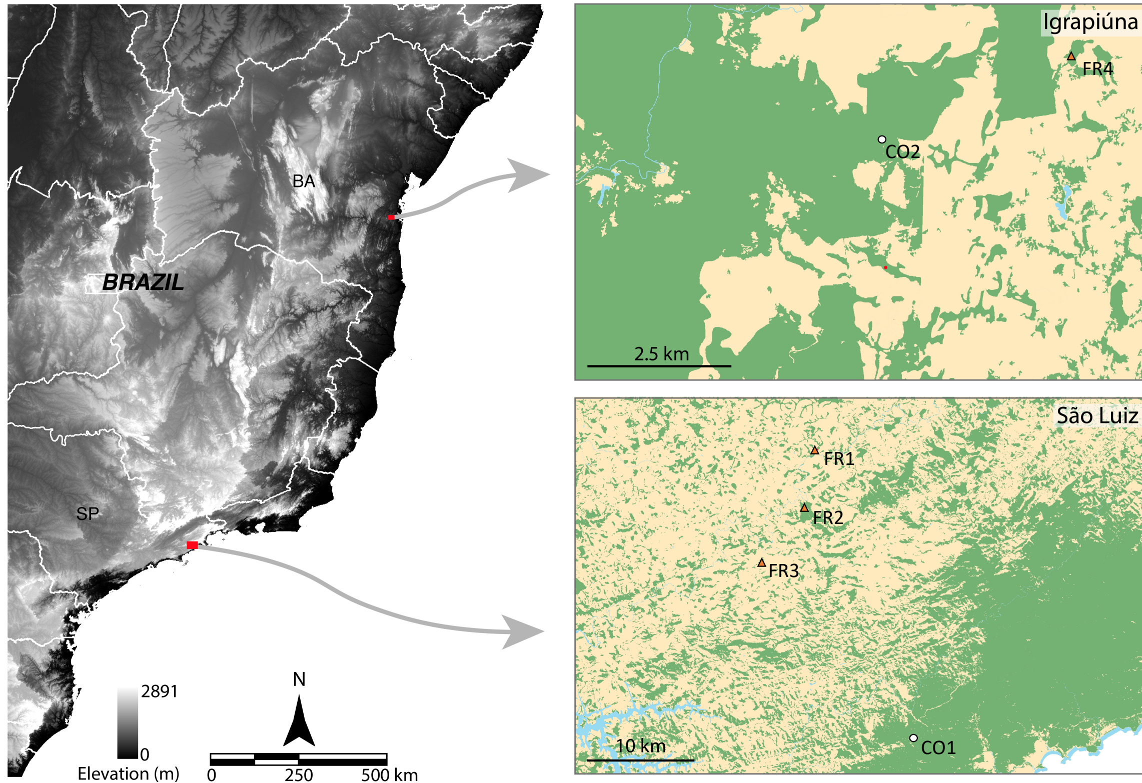


Figure 5-19: MHC IIB haplotype network for four focal species.
Circle size is proportional to haplotype frequency, colors correspond to the populations in which each haplotype is found, and the length of the links between haplotype circles correspond to the genetic distance between haplotypes.

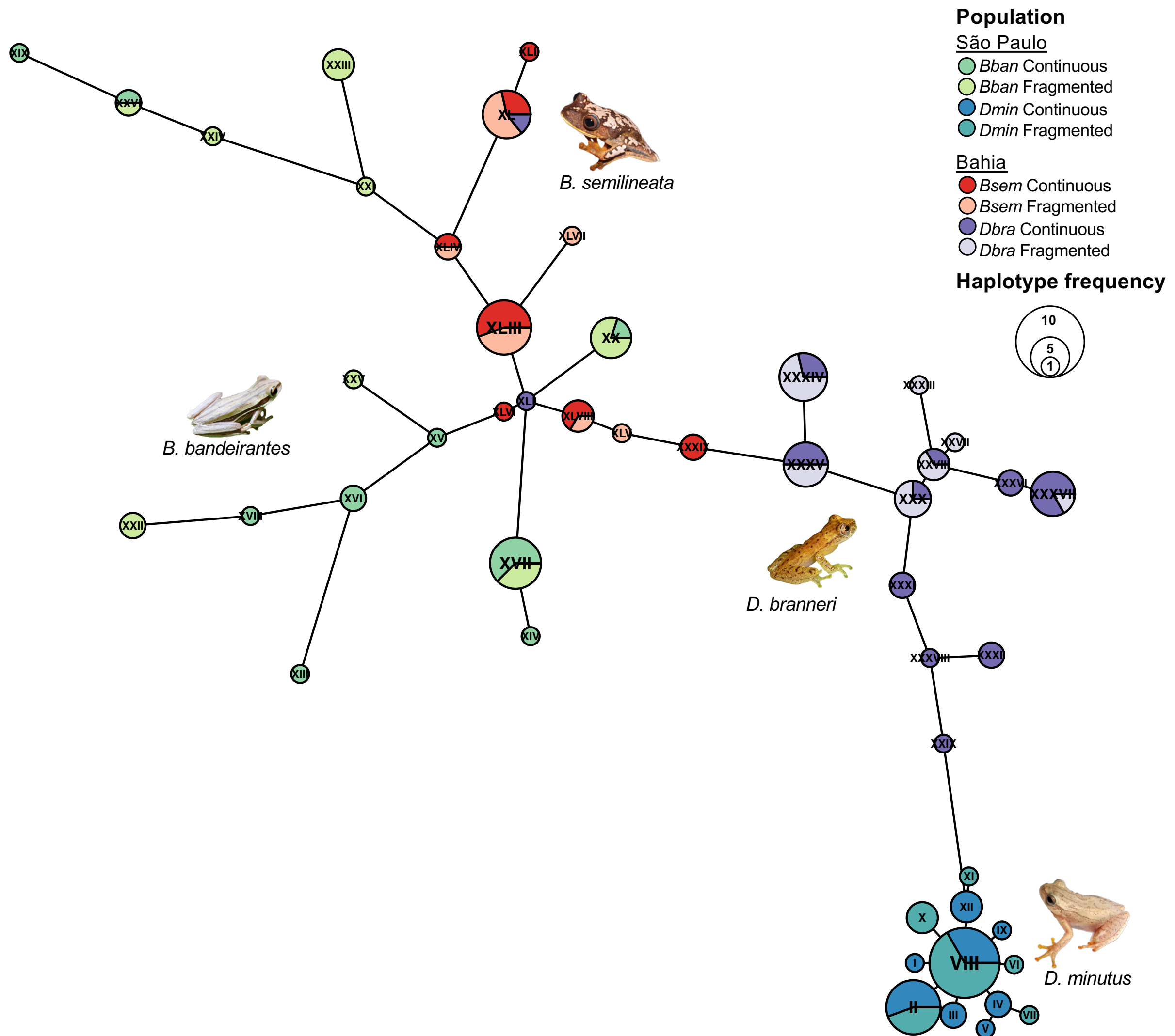


Figure 5-20: MHC IIB summary statistics across all focal species.
Sampling region (SP = São Paulo, BA = Bahia) is specified in parentheses after each species' name. **(A, B)** MHC IIB immunogenetic diversity erodes in fragmented populations by both expected heterozgosity (A) and nucleotide diversity (B). Dark green bars represent populations from continuous forests and light green bars represent populations from fragmented forests. Asterisks represent a significant difference according to t-tests (alpha = 0.05). **(C)** MHC IIB F_{ST} does not fall within the 95% Confidence Interval of any focal species. ddRAD F_{ST} mean values are shown by red circles with error bars and MHC IIB F_{ST} values are shown by blue squares.

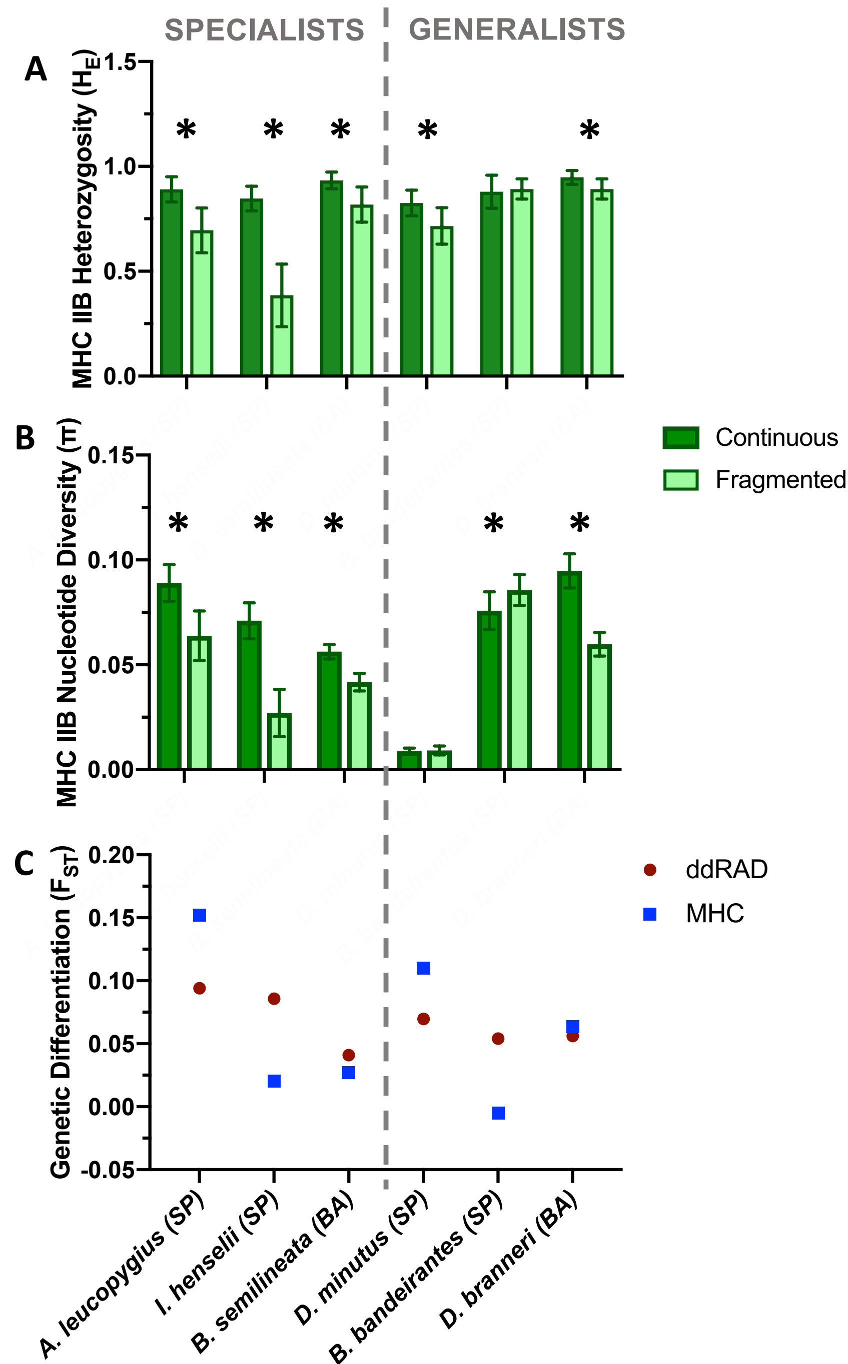


Figure 5-21: Relationships between MHC IIB Heterozygosity, overall genetic diversity, and infections.
(A) MHC IIB observed heterozygosity is negatively associated with overall observed heterozygosity estimated from ddRAD loci. **(B)** *Bd* prevalence (% of individuals infected with *Bd* in a population) is negatively associated with MHC IIB immunogene diversity (expected heterozygosity). **(C)** Apicomplexan load (% of ddRAD reads from Apicomplexa OTUs) is positively associated with MHC IIB immunogene diversity (expected heterozygosity).

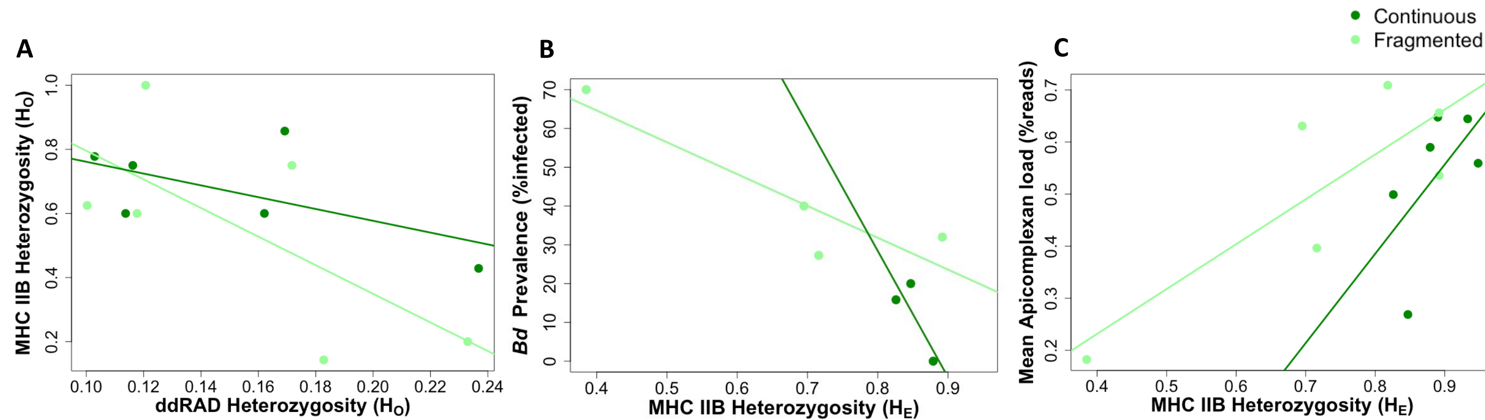


Figure 5-22: Pathogen incidence across populations.
(A) *Bd* prevalence in São Paulo was significantly higher in in fragmented than continuous forests and prevalence tended to be higher in specialists in both habitat types. **(B)** *Bd* infection loads tended to increase in fragmented habitats in specialists and did not change between habitat types in generalists. **(C)** Apicomplexan loads were significantly higher in Bahia than in São Paulo.

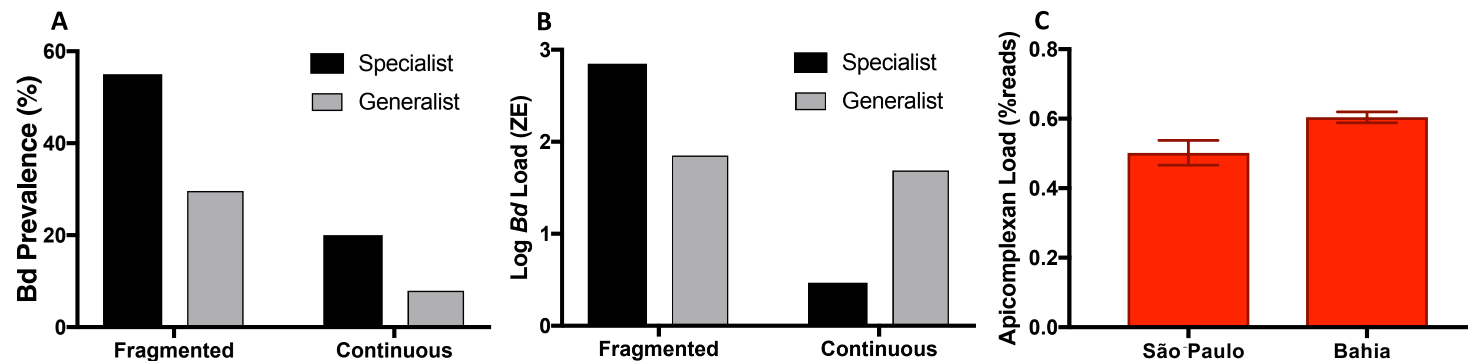
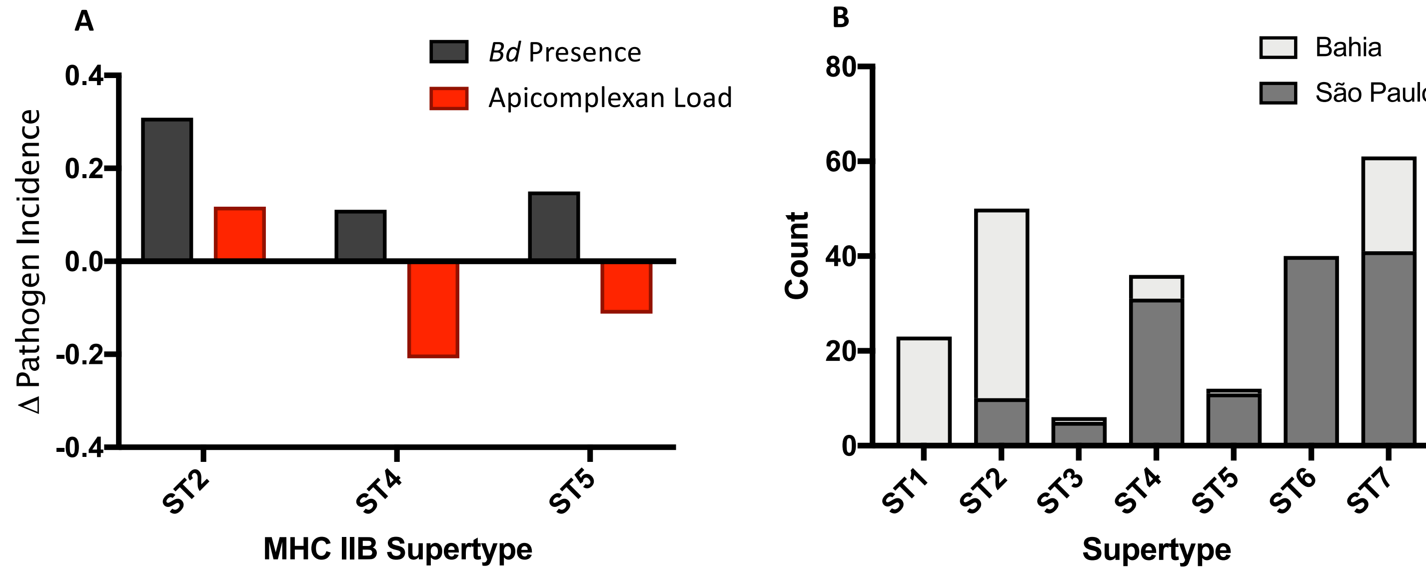


Figure 5-23: Relationships between MHC IIB Supertypes, pathogen incidence, and sampling regions.
(A) Change in pathogen incidence (number infected with *Bd* or Apicomplexan load) in the presence of three MHC IIB supertypes, ST2, ST4, and ST5. Frogs possessing ST2 were more likely to be infected with *Bd* and had higher Apicomplexan loads relative to those without ST2. Frogs possessing ST4 or ST5 were more likely to be infected with *Bd* but had lower Apicomplexan loads relative to those without these supertypes. **(B)** Distribution of the seven MHC IIB supertypes across the two sampling regions. ST1 was only found in *D. branneri* and ST6 was only found in *D. minutus*. ST4 and ST5 were relatively rare in Bahia, where Apicomplexan loads were higher.



Supplemental Figures

Figure 5-S24: Neighbor-joining tree of MHC IIB haplotypes across all species.
Tree was imputed using the BioNJ algorithm and HKY model. Nodes are labeled with abbreviations representing the species in which the haplotype is found (Aleu = *A. leucopygius*, Ihens = *I. henselii*, Dmin = *D. minutus*, Bban = *B. bandeirantes*, Bsem = *B. semilineata*, Dbra = *D. branneri*, Shared = shared between *B. semilineata* and *D. branneri*) followed by the haplotype name, and the supertype to which the haplotype belongs.

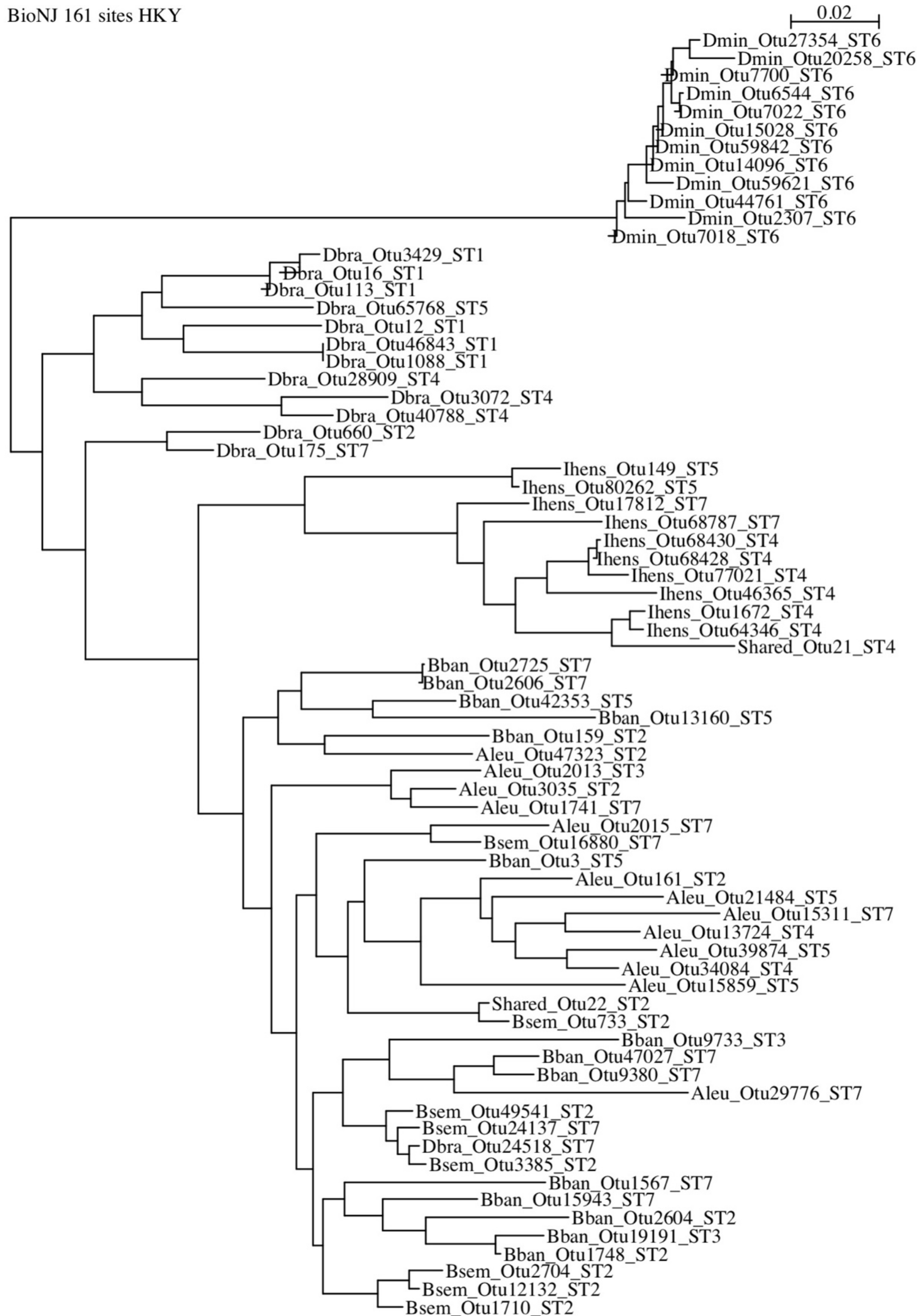


Figure 5-S25: Alignment of MHC IIB amino acid sequence showing pocket folding sites and positively selected sites.
Codon position numbers are listed for the current study, Bataille et al. 2015, and Mulder et al. 2017 alignments. Two amino acid sequence per species and the one shared haplotype (Otu22) are included from the current study, and two previously published sequences from Bataille et al. 2015 are included from each of four species (*Litoria verreauxii*, *Bombina bombina*, *Rana yavapaiensis*, and *Xenopus laevis*). Colors indicate positions corresponding with putative P9 (orange), P6/P7 (yellow), P4/P7 (green) and P4 (blue) pocket residues that correspond with pockets of the MHC II molecule peptide-binding groove. Sites that were found to be under positive selection and that were extracted to condense haplotypes into supertypes are bolded and enclosed in black boxes.

Current study codon position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	**27**	28	29	**30**	31	32	33	34	35	36	**37**	38	39	40	**41**	42	43	**44**	45	46	47	48	49	50
Bataille et al. 2015 position	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	**57**	58	59	**60**	61	62	63	64	65	66	**67**	68	69	70	**71**	72	73	**74**	75	76	77	78	79	80
Mulder et al. 2017 position	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	**52**	53	54	**55**	56	57	58	59	60	61	**62**	63	64	65	**66**	67	68	**69**	70	71	72	73	74	75
Dmin Otu59842	F	Y	N	Q	E	E	F	V	Y	F	D	S	D	V	G	K	Y	I	A	K	T	E	F	G	K	P	**V**	A	V	**Y**	Y	N	S	D	K	N	**Y**	I	D	Q	**M**	K	M	**R**	V	E	T	F	C	R
Dmin Otu27354	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	**.**	S	.	**.**	.	.	.	.	.	.	**.**	.	.	.	**.**	.	.	**.**	.	.	.	.	.	.
Dbra Otu16	L	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	S	**T**	.	D	**A**	W	.	.	N	.	D	**I**	.	.	.	**K**	R	S	**E**	.	.	.	V	.	K
Dbra Otu12	L	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	S	**T**	.	D	**G**	W	.	.	N	.	D	**I**	.	E	D	**R**	R	S	**S**	.	.	.	V	.	K
Ihen Otu68430	L	.	.	.	.	.	L	.	.	.	.	.	.	.	.	E	F	.	.	.	.	.	.	.	R	I	**Q**	.	D	**S**	W	.	K	N	.	E	**I**	L	A	D	**K**	R	S	**A**	.	.	.	V	.	K
Ihen Otu64346	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	R	I	**Q**	.	D	**N**	W	.	K	N	.	E	**I**	L	A	D	**E**	R	S	**.**	.	.	.	V	.	K
Bban Otu159	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	L	.	R	.	**Q**	.	E	**.**	L	.	K	N	.	D	**.**	.	E	.	**L**	.	S	**Y**	.	.	.	V	.	K
Bban Otu47027	L	.	.	.	.	.	.	M	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	L	.	R	.	**Q**	.	E	**.**	F	.	K	N	.	D	**L**	.	E	.	**R**	.	S	**A**	.	.	.	V	.	K
Aleu Otu1408	L	.	.	.	.	.	.	.	.	.	.	.	N	.	.	Y	.	.	.	.	.	.	.	.	.	.	**D**	.	D	**.**	W	.	K	N	.	D	**F**	.	E	E	**K**	R	S	**A**	.	.	.	V	.	K
Aleu Otu161	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	F	.	.	.	.	.	L	.	.	.	**S**	.	D	**.**	W	.	K	.	.	D	**F**	.	E	.	**K**	R	S	**A**	.	.	.	.	.	K
Bsem Otu1710	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	.	T	.	.	.	.	.	.	R	.	**D**	.	E	**.**	W	.	K	.	.	D	**F**	.	E	.	**R**	.	S	**V**	.	.	.	V	.	K
Bsem Otu2704	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	L	.	R	S	**A**	.	E	**.**	W	.	K	.	.	D	**F**	.	E	.	**R**	.	S	**V**	.	.	.	V	.	K
Shared Otu22	L	.	.	.	.	.	T	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	R	.	**T**	.	E	**.**	W	.	K	.	.	D	**F**	L	E	.	**Q**	R	S	**A**	.	.	.	V	.	K
Livea5a-R	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	R	.	**D**	.	D	**.**	W	.	N	N	.	D	**I**	.	E	.	**K**	.	S	**.**	.	.	.	V	.	K
Livea-S	S	.	.	A	.	.	Y	.	.	.	.	.	.	.	.	Y	F	.	G	.	.	.	I	.	R	Q	**Q**	.	D	**S**	W	.	K	N	.	D	**I**	.	E	.	**E**	.	S	**N**	.	.	.	V	.	K
BoboBeta14(EF 210771)	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	R	.	**D**	.	D	**.**	W	.	.	N	.	D	**I**	.	G	R	**A**	.	A	**A**	-	-	-	-	-	-
BoboBeta13(EF 210770)	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	R	.	**D**	.	D	**.**	W	.	.	N	.	D	**I**	.	E	R	**A**	.	A	**A**	-	-	-	-	-	-
RayaA (JN638850)	I	.	.	.	.	.	Y	.	.	.	.	.	.	R	.	F	F	.	P	R	.	.	Y	.	R	.	**D**	.	D	**.**	W	.	N	N	P	D	**I**	L	E	R	**V**	R	A	**A**	.	.	.	I	.	K
RayaB (JN638851)	I	.	.	.	.	.	Y	.	.	.	.	.	.	R	.	F	.	.	P	I	.	.	Y	.	R	L	**D**	.	D	**.**	W	.	N	N	P	D	**V**	L	E	R	**A**	R	A	**E**	.	.	R	V	.	K
XelaH1 (EF210754)	Y	.	.	L	.	.	T	D	.	.	.	.	.	.	.	L	F	.	.	.	.	.	L	.	.	.	**D**	.	D	**.**	W	.	N	Q	.	E	**F**	L	E	.	**K**	L	A	**E**	-	-	-	-	-	-
XelaG1 (EF210753)	Y	.	.	L	.	.	T	D	.	.	.	.	.	.	.	L	F	.	.	.	.	.	L	.	.	.	**S**	.	D	**.**	W	.	.	Q	.	D	**F**	L	E	.	**T**	R	A	**A**	-	-	-	-	-	-

KEY
P9
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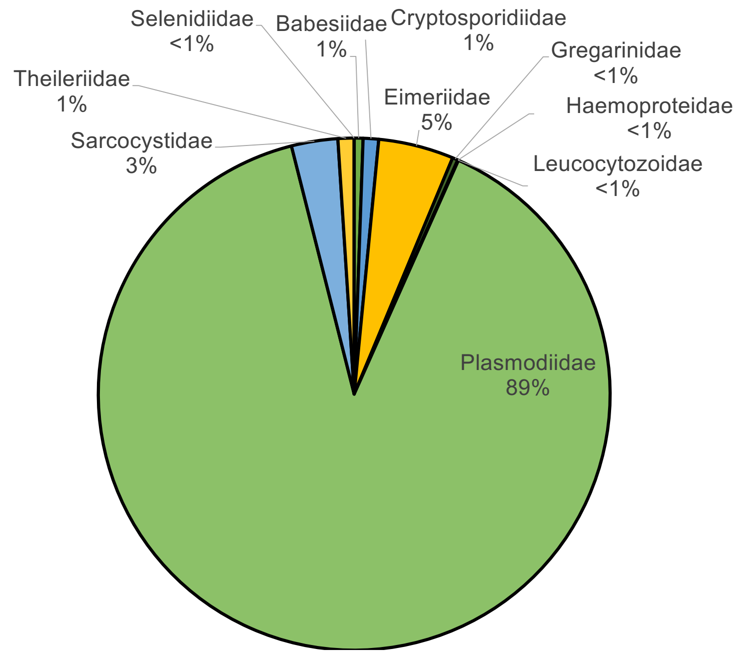
P4

Figure 5-S26: Taxonomic diversity of Apicomplexan OTUs found in ddRAD sequences from the six focal species.



Chapter 6: Conclusion

In the four data chapters of my dissertation, I evaluated the effects of habitat fragmentation on genetic diversity and potential disease susceptibility in frogs of the Brazilian Atlantic Forest. I used three systems to examine factors that are rarely considered simultaneously in studies of fragmentation and disease: time (long-term vs. short-term), species ecology (habitat generalist vs. specialist), and landscape context (intensive vs. rustic land-use). By comparing these systems and a variety of focal species, my research deepens our understanding of the range of expected impacts due to habitat fragmentation, and provides baseline data for further studies.

In Chapter 2, I compared inbred island populations to mainland populations of a single frog species, *Thoropa taophora*. I found that contrary to our expectations, immunogenetic diversity at the MHC IIB locus has sharply declined with overall genetic diversity in fragmented island populations. Although the focal species I selected across the study area did not harbor significant infections of my original target pathogen *Batrachochytrium dendrobatidis* (*Bd*), I was able to assess infections by a variety of eukaryotic microbes found on frog skin using high-throughput sequencing of skin swabs. I found that inbred populations hosted proportionally more potential eukaryotic parasites, and across all populations I detected an MHC heterozygote advantage, whereby heterozygotes hosted proportionally fewer potential eukaryotic parasites. When only mainland frogs were analyzed to remove confounding effects of environmental differences between mainland and island sites contributing to this pattern, I found that immunogenotype still explained the proportion of skin microbes that were potentially parasitic.

In Chapter 3, I explored the overall skin microbiome across these *Thoropa taophora* populations to examine how microbiome diversity and assembly is related to both long-term fragmentation and host genetic diversity and immunogenotype. While previous studies have largely focused on the bacterial component of the amphibian skin microbiome, I simultaneously analyzed bacteria and microeukaryotes inhabiting frog skin. I found that microbiome diversity was directly related to host genetic diversity and host immunogenotype. I also recovered two novel patterns when I compared the bacterial and eukaryotic communities in the skin microbiome: (1) bacterial and eukaryotic diversity were positively correlated; and (2) the microbiome network is composed of cross-Domain connections (between bacteria and eukaryotes). Currently one of the most compelling and widely discussed treatments for amphibian disease is probiotic treatment – manipulating the skin microbiome with bacteria that fight pathogens. However, my results suggest that altering the bacterial community may significantly alter the eukaryote community found on frog skin. We know very little of the ecological roles these eukaryotes play in frog health and in biotic interactions between frogs and other species. My results imply that more thorough research is needed before these probiotic strategies should be widely employed in wild populations.

Although the patterns I recovered from the island-mainland system are intriguing and provide insights into time-lagged effects of contemporary fragmentation, the results from this system may have limited relevance to current conservation strategies to deal with anthropogenically fragmented wildlife populations. Therefore, I moved to the Brazilian Atlantic Forest mainland for the remainder of my research. On the mainland I was able to sample a larger number of focal species relative to the species-poor island and coastal environments, allowing me to examine how frog species ecology influences the impacts of fragmentation. The mainland

also has fragmented and modified habitats across a range of land-use types, from intensive (open managed pasture) to rustic (agroforest). This allowed me to examine how the impacts of fragmentation vary according to land-use context.

In Chapter 4, I examined the impacts on overall genetic diversity in frog species that vary in ecology (high-dispersing habitat generalists vs. low-dispersing habitat specialists) and that inhabit modified landscapes that vary in their land-use (intensive cattle pasture vs. rustic cacao agroforests). I hypothesized that all frog species would be impacted by fragmentation but to different extents. Specifically, I predicted that all frogs would lose genetic diversity and exhibit genetic isolation due to fragmentation, but that these effects would be more pronounced in specialists. This hypothesis was partly supported: I found that while generalists lost genetic diversity with fragmentation, specialists showed signatures of genetic isolation. The most surprising result was that specialists actually exhibited *higher* genetic diversity in fragmented habitats. However, after I researched the phylogeography of frogs in this region, I found that this result is consistent with high levels of genetic structure in forest specialists compared with habitat generalists. This means that specialists in fragmented populations are likely the result of a mixture of source populations that show relict genetic diversity due to historical population structure, whereas continuous habitat specialist populations likely represent a single population that has been stable for a long time. Generalists show very little historical structure, and thus it makes sense that genetic differentiation would be lower across the landscape.

The second hypothesis I tested in Chapter 4 was that rustic forms of agriculture could buffer populations against loss of genetic diversity and dispersal. Again, this hypothesis was partly supported – in terms of genetic diversity, I saw equal or higher genetic diversity in rustic modified landscapes relative to continuous preserved forest. However, I observed genetic

isolation between a forest and a rustic landscape in the specialist focal species from this region. This suggests that there may be barriers to dispersal or local adaptation in rustic landscapes, but that these landscapes can nonetheless support genetically diverse frog populations.

Finally, in Chapter 5, I examined the effects of habitat fragmentation on frog immunogenetics and infections across the ecologically diverse focal species. I found that immunogenetic diversity is eroded in fragmented populations, and more severely in forest specialists than in habitat generalists. After comparing immunogenetic data with genome-wide genetic data, I concluded that selection is likely contributing to the loss of immunogenetic diversity in fragmented populations rather than solely inbreeding and drift.

I also tested these populations for infections by *Bd* and apicomplexan parasites. I hypothesized that higher elevation São Paulo populations would exhibit higher *Bd* infections while apicomplexan infections would be more significant in lowland Bahia, where habitat suitability for *Bd* is lower. My findings supported this hypothesis. I also used pathogen data to test whether fragmentation increases infection susceptibility, and indeed I found that both *Bd* prevalence and apicomplexan loads were higher in fragmented populations. Finally, I evaluated the relationships between the MHC IIB locus and infections. I recovered an MHC IIB heterozygote advantage for *Bd* but not for Apicomplexans. In fact, both *Bd* and Apicomplexans were associated with MHC heterozygosity, but in opposite directions: higher heterozygosity was associated with higher *Bd* prevalence but lower apicomplexan loads. It is unclear whether this is a direct result of heterozygote advantage in *Bd* only, or competitive release for Apicomplexans when *Bd* is lower or absent.

When I evaluated the relationships between these pathogens and MHC IIB supertype, I found that three MHC supertypes were significantly associated with infections. One Supertype

showed a tradeoff where *Bd* prevalence is higher but apicomplexan loads are lower. As this is a correlative study, it is not certain that MHC is associated with differences in Apicomplexan diversity. It could be that *Bd* and Apicomplexans "compete" in a sense for host resources or interact indirectly through the host immune system, rather than that both are impacted by MHC molecules in the same way. Regardless, my study shows that fragmentation can shift immunogenetic diversity in ways that impact infections.

Taken together, my results imply that habitat fragmentation increases disease susceptibility in amphibians. The value of my research lies in the focus on natural populations, however this also its limitation: all of the research I have presented in my dissertation is based on correlative studies of wild populations. Further studies are needed to provide evidence of causal relationships, to demonstrate *e.g.*, pathology of the potential parasites I identified in Chapter 2, causal relationships between MHC IIB genotype and the assembly of the microbiome that my results implied in Chapter 3, and mechanistic interactions between MHC IIB genotype and Apicomplexan parasites that are suggested by my results from Chapter 5. In addition, my results from Chapter 4 suggest that genetic diversity may not be compromised by fragmentation in sensitive forest specialist frogs, however it remains unknown how the absolute level of genetic diversity impacts population resilience in these species. Nonetheless, my dissertation research provides evidence that habitat fragmentation impacts genetics and potential disease susceptibility in frogs. Future research and conservation efforts should consider a range of species, pathogens, and landscape contexts to gain a holistic picture of land-use impacts on health in wildlife populations.

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Appendix

Appendix 2-1: Potentially parasitic OTUs recovered from Thoropa taophora skin swabs. OTUs were obtained through Next Generation Sequencing (Illumina MiSeq) of the eukaryotic 18S v4 genetic region. Likely ecology was obtained through a standardized Google Scholar search of peer-reviewed literature.

OTU id	No. Reads	No. Swabs	Top BLAST hit	e-value	ID %	Higher classification	Likely ecology	Citation
New.CleanUp.ReferenceOTU2449	368	6	Anurofeca richardsi	1.00E-169	96%	Ichthyosporea	amphibian larva pathogen	Rowley et al. 2013
New.ReferenceOTU112	9681	8	Hepatozoon sp.	<2.23E-308	99%	Apicomplexan, haemagregarine	amphibian parasite	Desser et al. 1995
New.CleanUp.ReferenceOTU912	680	1	Perkinsus qugwadi	2.00E-51	83%	Dinoflagellate, Perkinsidae	animal parasite	Chambouvet et al. 2015
AB272236	241	2	Strongyloides sp.	5.00E-173	98%	Nematoda, Strongyloididae	animal parasite (known from amphibians)	Patterson-Kane et al. 2001
AF293895	245	8	Echinamoeba exundans	<2.23E-308	100%	Amoeba	animal pathogen	Wannasan 2013
New.CleanUp.ReferenceOTU1824	1665	1	Cryptosporidium struthionis	1.00E-111	86%	Apicomplexan	animal pathogen	Densmore and Green 2007
New.ReferenceOTU0	1343	1	Cryptosporidium struthionis	6.00E-153	94%	Apicomplexan	animal pathogen	Densmore and Green 2007
EF023236	24	1	Eimeriidae	<2.23E-308	100%	Apicomplexan	animal pathogen	Densmore and Green 2007
EF024722	25	1	Eimeriidae	2.00E-126	85%	Apicomplexan	animal pathogen	Densmore and Green 2007

New.CleanUp.ReferenceOTU953	864	5	Schellackia or Eimeria	2.00E-76	78%	Apicomplexan, Coccidia	animal pathogen	Densmore and Green 2007
New.ReferenceOTU169	846	1	Adalina dimidiata	7.00E-159	93%	Apicomplexan, Coccidia, Adeleidae	animal pathogen	Densmore and Green 2007
DQ096836	1298	3	Adelina bambarooniae	<2.23E-308	99%	Apicomplexan, Coccidia, Adeleidae	animal pathogen	Densmore and Green 2007
New.CleanUp.ReferenceOTU707	218	2	Eimeria percae	4.00E-105	85%	Apicomplexan, Coccidia, Eimeriidae	animal pathogen	Densmore and Green 2007
New.CleanUp.ReferenceOTU693	178	1	Eimeria quokka	1.00E-80	78%	Apicomplexan, Coccidia, Eimeriidae	animal pathogen	Densmore and Green 2007
New.ReferenceOTU38	1773	1	Goussia chalupskyi	9.00E-69	77%	Apicomplexan, Coccidia, Eimeriidae	animal pathogen	Densmore and Green 2007
AF368504	997	11	Basidiobolus sp.	6.00E-152	100%	Entomophthoro-mycota	animal pathogen	Whitaker 2016
New.CleanUp.ReferenceOTU384	2470	6	Conidiobolus coronatus	3.00E-170	98%	Entomophthoro-mycota	animal pathogen	Gugnani 1992
New.ReferenceOTU22	60	1	Conidiobolus nanodes	1.00E-137	86%	Entomophthoro-mycota	animal pathogen	Gugnani 1992
New.ReferenceOTU180	83	2	Hepatozoon sp.	<2.23E-308	97%	Apicomplexan, Coccidia	animal pathogen (known from amphibians)	Densmore and Green 2007
AF293902	48	1	Saccamoeba limax	<2.23E-308	97%	Amoeba, Hartmannellidae	animal skin pathogen	Wannasan 2013
New.CleanUp.ReferenceOTU1144	122	4	Saccamoeba limax or Ptolemeba bulliensis	1.00E-164	94%	Amoeba, Hartmannellidae	animal skin pathogen	Wannasan 2013
New.ReferenceOTU85	506	2	Saccamoeba sp.	4.00E-169	97%	Amoeba, Hartmannellidae	animal skin pathogen	Wannasan 2013

AM743095	488	12	Vermamoeba vermiformis	<2.23E-308	99%	Amoeba, Hartmannellidae	animal skin pathogen	Wannasan 2013
New.CleanUp.ReferenceOTU1891	240	3	Ichthyosporea sp.	2.00E-172	96%	Ichthyosporea	aquatic animal parasite	Rowley et al. 2013
M32705	95	3	Thraustotheca clavata or Achlya sp.	<2.23E-308	100%	Stramenopile, Oomycete, Saprolegniaceae	aquatic animal parasite	Gleason et al. 2014
AF300282	1035	6	Chilodonella uncinata	<2.23E-308	100%	Ciliate	aquatic animal pathogen	Langdon et al. 1985
AY835669	164	4	Pseudocohnilembus persalinus	<2.23E-308	100%	Ciliate	aquatic animal pathogen	Kim et al. 2004
New.CleanUp.ReferenceOTU593	504	3	Malassezia furfur	2.00E-103	83%	Basidiomycota, Ustilagino-mycotina	commensal, maybe opportunistic	Matousek et al. 2002
DQ118537	183	1	Raillietnema sp.	<2.23E-308	99%	Nematode, Cosmocercidae	frog gut parasite	Bursey and Goldberg 2006
New.CleanUp.ReferenceOTU505	25	2	Parapharyngodon echinatus	5.00E-25	72%	Nematode, Pharyngodonidae	frog parasite	Dyer et al. 1995
New.CleanUp.ReferenceOTU1758	109	2	Proteromonas lacertae	5.00E-104	87%	Stramenopile, Slopalinida	hemogregarine parasite	Maia et al. 2012
GU647190	39	1	Acanthoeca spectabilis or Acanthocoepsis unguiculata	8.00E-122	88%	Choanoflagellate	likely novel fish pathogen	Kerk et al. 1995
EU011929	69	4	Salpingoeca sp.	3.00E-180	97%	Choanoflagellate	likely novel fish pathogen	Kerk et al. 1995
New.CleanUp.ReferenceOTU613	36	3	Salpingoeca tuba	4.00E-60	87%	Choanoflagellate	likely novel fish pathogen	Kerk et al. 1995
New.CleanUp.ReferenceOTU678	48	2	Blastocystis sp.	4.00E-135	88%	Stramenopile	opportunistic animal gut pathogen	Stensvold et al. 2009

AF293898	246	2	Leptomyxa reticulata	<2.23E-308	98%	Amoeba	opportunistic animal pathogen	Weiss et al. 1996
EF141325	94	2	Yarrowia lipolytica	2.00E-162	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Liu et al. 2013
New.CleanUp.ReferenceOTU640	56	2	Yarrowia sp.	8.00E-42	78%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Liu et al. 2013
AB018141	230	2	Candida allociferrii	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
AB013559	254	4	Candida apicola	<2.23E-308	99%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
New.CleanUp.ReferenceOTU387	31	1	Candida bromeliacearum	3.00E-26	75%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
New.CleanUp.ReferenceOTU1946	43	1	Candida deformans	4.00E-155	99%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
AY198400	57	3	Candida edaphicus	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
AB018142	193	3	Candida etchellsii	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
New.CleanUp.ReferenceOTU2202	119	11	Candida glucosophila	<2.23E-308	99%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
AB013572	4383	34	Candida haemulonis 2	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
AB013548	19795	28	Candida heliconiae	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
New.CleanUp.ReferenceOTU2135	83	2	Candida mogii	2.00E-141	92%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
EF141326	24825	50	Candida parapsilosis	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014

EF550483	73	5	Candida peoriensis or C. odintsovae	6.00E-179	98%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
New.CleanUp.ReferenceOTU1858	2414	22	Candida sp.	<2.23E-308	98%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
New.CleanUp.ReferenceOTU2317	30	1	Candida sp. 2	4.00E-174	96%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
New.CleanUp.ReferenceOTU940	43	1	Candida sp. 3	2.00E-178	97%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
New.CleanUp.ReferenceOTU1350	85	1	Candida sp. 4	4.00E-175	96%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
New.CleanUp.ReferenceOTU1477	219	3	Candida sp. 5	2.00E-126	90%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
AB018175	122	1	Candida stellata	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
AB013513	132074	81	Debaryomyces or Kluyveromyces marxianus	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Deorukhkar et al. 2014
AB083080	292	1	Dipodascus capitatus	5.00E-160	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Ersoz et al. 2004
X69842	99	2	Geotrichum candidum	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Verghese and Ravichandran 2003
AB053245	961	7	Issatchenkia terricola	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Han et al. 2004
AEWP01001287	21	16	Kazachstania sp.	5.00E-163	99%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Jenney et al. 2004
New.ReferenceOTU60	25230	8	Kluyveromyces or Saccharomyces or Kazachstania	2.00E-177	97%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Jenney et al. 2004
GQ120116	477	2	Kodamaea ohmeri*	6.00E-178	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Han et al. 2004

Y12511	66	1	Ogatea or Pichia	<2.23E-308	99%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Han et al. 2004
AB054569	49	2	Pichia jadinii	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Han et al. 2004
AY251635	21528	17	Pichia or Issatchenkia	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Han et al. 2004
New.CleanUp.ReferenceOTU23	192	4	Pichia scolyti	2.00E-177	98%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Han et al. 2004
AAZN01000374	3584	62	Saccharomyces cerevisiae	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Enache-Agoulvant and Hennequin 2005
New.CleanUp.ReferenceOTU2338	99	2	Saccharomyces sp.	<2.23E-308	100%	Ascomycota, Saccharomycete	opportunistic animal pathogen	Enache-Agoulvant and Hennequin 2005
AB001758	21	3	Trichosporon terricola	<2.23E-308	100%	Basidiomycota	opportunistic animal pathogen	Hoy et al. 1986
AY520284	54	5	Trichosporon sp.	<2.23E-308	100%	Basidiomycota	opportunistic animal pathogen	Hoy et al. 1986
AJ515169	2341	44	Cryptococcus perniciosus or nemorosus	<2.23E-308	100%	Basidiomycota, Agaricomycotina	opportunistic animal pathogen	Jenney et al. 2004
New.CleanUp.ReferenceOTU915	23	1	Cryptococcus sp.	8.00E-177	97%	Basidiomycota, Agaricomycotina	opportunistic animal pathogen	Jenney et al. 2004
New.ReferenceOTU27	1945	27	Cryptococcus sp.	<2.23E-308	100%	Basidiomycota, Agaricomycotina	opportunistic animal pathogen	Jenney et al. 2004
AB032617	153	10	Cryptococcus sp. (maybe albidus)	<2.23E-308	100%	Basidiomycota, Agaricomycotina	opportunistic animal pathogen	Jenney et al. 2004

New.ReferenceOTU131	20	1	Leucosporidium fellii	2.00E-178	97%	Basidiomycota, Puccinio-mycotina	opportunistic animal pathogen	Lanteri et al. 2012
New.ReferenceOTU183	1858	31	Rhodotorula diobovata or Rhodosporidium paludigenum	<2.23E-308	100%	Basidiomycota, Puccinio-mycotina	opportunistic animal pathogen	Sutton et al. 2016
AB126648	607	21	Rhodotorula or Cystobasidium	<2.23E-308	99%	Basidiomycota, Puccinio-mycotina	opportunistic animal pathogen	Sutton et al. 2016
New.ReferenceOTU114	280	8	Malassezia japonica	<2.23E-308	100%	Basidiomycota, Ustilagino-mycotina	opportunistic animal pathogen	Seyedmousavi et al. 2015
AAXK01002636	2369	97	Malassezia globosa	<2.23E-308	100%	Basidiomycota, Ustilagino-mycotina	opportunistic animal pathogen	Seyedmousavi et al. 2015
New.CleanUp.ReferenceOTU902	20	1	Halicephalobus cf. gingivalis	2.00E-93	85%	Nematode, Panagrolaimidae	opportunistic animal pathogen	Loo et al. 2015
AF396684	854	6	Aphanomyces sp.	<2.23E-308	99%	Stramenopile, Oomycota, Saprolegniales	opportunistic animal pathogen	Gleason et al. 2014
New.CleanUp.ReferenceOTU1541	2424	15	Protoopalina intestinalis	7.00E-83	80%	Stramenopile, Opalinidae	opportunistic animal pathogen	Densmore and Green 2007
New.CleanUp.ReferenceOTU1903	2582	21	Protoopalina intestinalis	6.00E-96	82%	Stramenopile, Opalinidae	opportunistic animal pathogen	Densmore and Green 2007
FJ794932	240	5	Pythium sp.	<2.23E-308	99%	Stramenopile, Oomycota	opportunistic animal pathogen?	Gleason et al. 2014
AB126047	69	1	Sporobolomyces diospyroris	<2.23E-308	98%	Basidiomycota, Puccinio-mycotina	opportunistic animal skin pathogen	Bergman and Kauffman

New.CleanUp.ReferenceOTU515	415	3	Opisthostyla sp.	2.00E-171	97%	Ciliate	opportunistic frog pathogen	Pritchett and Sanders 2007
GQ872428	311	4	Opisthostyla sp. or Epistylis riograndensis	<2.23E-308	99%	Ciliate	opportunistic frog pathogen	Pritchett and Sanders 2007
New.CleanUp.ReferenceOTU1270	47	1	Hartmannella cantabrigiensis	<2.23E-308	100%	Amoeba	opportunistic keratin parasite	Abedkhojasteh et al. 2013
New.CleanUp.ReferenceOTU1694	103	1	Hartmannella sp.	3.00E-88	80%	Amoeba	opportunistic keratin parasite	Abedkhojasteh et al. 2013
New.ReferenceOTU167	752	1	Nolandella (Hartmannellidae)	3.00E-75	77%	Amoeba	opportunistic keratin parasite	Abedkhojasteh et al. 2013
New.CleanUp.ReferenceOTU2271	40	1	Tetrahymena rostrata	2.00E-177	99%	Ciliate	opportunistic pathogen	Densmore and Green 2007
New.CleanUp.ReferenceOTU2181	26	1	Tetrahymena sp.	6.00E-172	98%	Ciliate	opportunistic pathogen	Densmore and Green 2007
X54512	108	3	Tetrahymena sp.	<2.23E-308	99%	Ciliate	opportunistic pathogen	Densmore and Green 2007
AJ972862	80	1	Rhinocladiella sp.	<2.23E-308	99%	Ascomycota	opportunitistic animal skin pathogen	Badali et al. 2010