Population Structure and Evolutionary Ecology of *Batrachochytrium dendrobatidis* in the Brazilian Atlantic Forest

by

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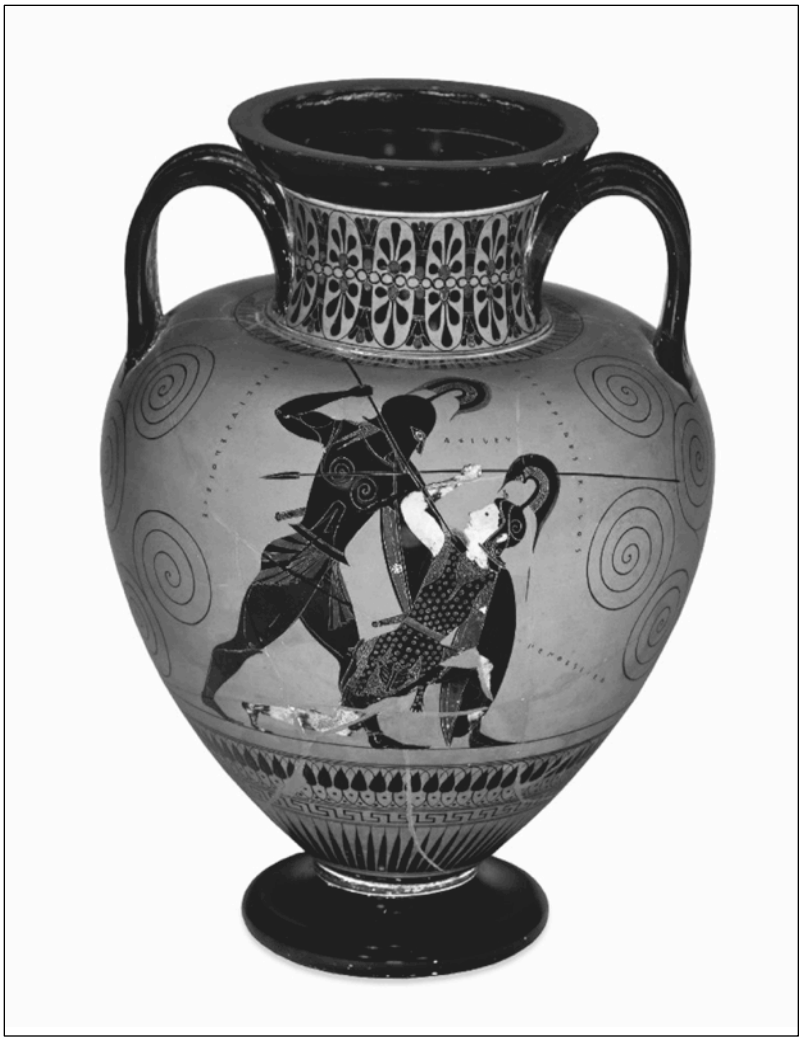
chytra (Greek) = pot or jar
The characteristic shape for which chytridiomycete fungi (chytrids) are named



Achilles killing Penthesilea
Greek jar, Athens (540-530 BC)
Photograph © Trustees of the British Museum [1]

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Abstract

The pathogen *Batrachochytrium dendrobatidis* (*Bd*), also known as the amphibian-killing chytrid fungus, causes the emerging disease implicated in recent population declines and extinctions of frog species worldwide. By taking advantage of unique *Bd* genetic diversity in the Brazilian Atlantic Forest this dissertation seeks to better understand the pathogen population history of the region, and to learn what ecological processes shape current – and future – distributions of this ecologically consequential pathogen. Data from prior infection studies show that the global *Bd* pandemic is caused by a single clonal genotype that is hypervirulent to amphibian hosts. In 2012, however, novel, divergent strains were reported from the Atlantic Forest region of southern Brazil. To investigate the distribution and population structure of this novel lineage, pure isolates of pathogen strains were collected from *Bd* infected anurans along a 2400 Km transect of the historical range of the Atlantic rainforest over three field seasons, The population genetic structure suggested the presence of a long-term endemic Brazilian lineage, and a recently introduced, invasive lineage, with an active hybrid zone that has formed in the southern Atlantic Forest where the two lineages come into contact. The discovery of the Brazilian hybrid zone is significant because *Bd* was once thought to be a strictly asexual pathogen, and the only known cases of hybridization in this species are restricted to this narrow zone on the Atlantic coast of Brazil. Natural hybrids present a valuable opportunity to understand the genetic basis of lineage divergence, and the first steps toward speciation. To understand the genetic differentiation between the divergent parental lineages, Illumina whole-genome sequences were obtained for 51

Brazilian Atlantic Forest strains of *Bd* – including three hybrids and representatives of both parental populations. Loci with unequally inherited alleles occurred in clustered blocks throughout the hybrid genomes. Gene ontology analyses showed that these divergent loci were enriched for genes responding to oxidative stress, suggesting a potential mechanism underlying the ecological diversification between *Bd* lineages. To investigate the competitive differences between *Bd* lineages, I assessed the competitive performance of *Bd*-Brazil against *Bd*-GPL *in-vivo* using a model amphibian host (*Hymenochirus curtipes*). Competition trials were performed using four different pairwise combinations of *Bd*-GPL and *Bd*-Brazil strains. The dominant strains at regular time points throughout the experiment were assessed by digital PCR using strain specific fluorescent probes. Competitive effects are observed between lineages, with *Bd*-GPL as the superior competitor, especially at the earlier stages of infection.

Chapter 1

Introduction: The amphibian-killing chytrid fungus

Over a third of the nearly 7,000 amphibian species known to science are threatened with extinction (IUCN 2015). This is the largest proportion of any biological class of organisms whose survival is at risk (Stuart *et al*. 2004). The welfare of these amphibians is intricately tied to our global ecosystems, and their decline will result in major changes to the biosphere. Amphibians provide critical, but often under appreciated ecosystem services. These include the control of insects which can transmit human diseases or act as agricultural pests, and serving as important prey items in the trophic networks of a variety of habitats (Valencia-Aguilar *et al*. 2013).

Amphibians already face the risk of extinction through deforestation, habitat loss, exposure to pesticides, and a changing climate; but none of these threats have been as acute or immediate as the recent spread of *Batrachochytrium dendrobatidis* (*Bd*). The current global outbreak of *Bd* has become the single greatest threat to an already imperiled group of organisms (Skerratt *et al*. 2007; Wake & Vredenburg 2008). This dissertation aims to clarify the historical relationships between the divergent strains of *Bd* in the Brazilian Atlantic Forest – a critical region of amphibian biodiversity – and shed light on the future trajectory of *Bd* evolution in the region. The following chapters are united in their focus on how pathogen population dynamics,

hybridization, and strain competition are shaping the evolution of this ecologically important pathogen.

History of the chytridiomycosis panzootic

In the late 1980s independent teams of herpetologists began to document sudden and enigmatic mass mortalities in amphibian populations from disparate regions of the globe (Berger *et al*. 1998). These population declines were occurring near simultaneously in seemingly unrelated localities. Many of the initial declines took place in pristine or protected environments ruling out habitat degradation as a cause of the die offs (*e.g.* the Monteverde Biological Reserve in central Costa Rica; Richards-Hrdlicka 2013; and a protected National Park in the mountains of El Copé, Panama; Ryan *et al*. 2008). It was not until 1998 that the cause of these declines was conclusively attributed to a globally spreading fungal pathogen. In 1999, this new chytrid pathogen was formally described as *Bd* (Longcore *et al*. 1999).

Bd attacks the keratinized epithelial tissues of amphibians and impairs skin functions that can lead to death in some species (Voyles *et al*. 2011). The skin is an especially important organ in amphibians because of its role in osmoregulation and facilitating gas exchange to supplement lung function (Van Rooij *et al*. 2015). Not all amphibian species are equally susceptible to *Bd*. Some amphibian species, most notably the North American bullfrog *Lithobates catesbeianus*, and the common laboratory model species, the African clawed frog *Xenopus laevis* are able to carry the pathogen without major disease symptoms (Garner *et al*. 2006). Carrier species like *L. catesbeianus* and *X. laevis* can act as possible vectors for the transmission of *Bd* to susceptible

populations (Daszak *et al*. 2004). *Bd* is now known to infect over 500 species of amphibians, and occurs on all continents except Antarctica, where no amphibian hosts occur (Olson *et al*. 2013).

Like its fellow members of the fungal phylum Chytridiomycota, *Bd* is fully aquatic and disperses via a motile spore (termed a zoospore) with a single posterior flagellum (Figure 1-1a). Most of the early diverging clades in the fungal kingdom have retained this flagellated spore stage as a means of dispersal (James *et al*. 2006). Unlike other chytrids, *Batrachochytrium* is the only described lineage that is pathogenic to terrestrial vertebrates. By contrast, other chytrids in the order Rhizophydiales fill ecological roles such as: plant and algal decomposition and plant/invertebrate pathogenesis. The closest known sister lineage to *Batrachochytrium* is *Homolaphlyctis polyrhiza* (also Rhizophydiales) a cellulose degrading saprotroph (Longcore *et al*. 2011).

Prior to the discovery of *Bd*, chytrid fungi were an obscure group, and an unlikely subject of research for all but a few investigators. The discovery of *Bd*, however, launched this relatively unknown, and critically understudied group into the focus of a global environmental crisis (James *et al*. 2009). The identification and phylogenetic placement of *Bd* in 1999 would not have been possible without development of the methods to isolate *Bd* in pure culture. The culture techniques developed in 1999 were largely adapted from established protocols for the field isolation and culture of other chytrid fungi, with modifications through trial and error (J. E. Longcore, *personal communication*).

The first isolate of *Bd* was collected from a blue poison dart frog (*Dendrobates tinctorius* var. *azureus*) in an infected colony at the Smithsonian National Zoo in Washington D.C. (Longcore *et al*. 1999). In 2004, widely adopted molecular diagnostic protocols for *Bd* based on real-time quantitative PCR were developed (Annis *et al*. 2004, Boyle *et al*. 2004). This real time PCR approach is highly reproducible and uses non-lethal skin swabs to detect *Bd.* It is the most widely used data collection method in *Bd* research, and its widespread employment has provided a clearer picture of where *Bd* occurs globally, and at what prevalence (Olson *et al*. 2013). These real time PCR assays depend on high copy repeats of the nuclear ribosomal internal transcribed spacer sequence (ITS). The high marker copy numbers are excellent for detecting trace amounts of pathogen DNA, but marker variation among copies prevent the clear inference of population genetic patterns.

To date, population genetic studies of *Bd* have largely relied on cultured material to produce the quantity of DNA needed for sequencing informative, single copy loci – or increasingly, whole genomes. The first population genetic study was published in 2003 (Morehouse *et al*. 2003), and was based on 5 variable loci and in 35 isolates. More recently Schloegel *et al*. (2012) used 35 loci to demonstrate the existence of a novel Brazilian endemic *Bd* clade and possible intra-specific hybridization between lineages. The Broad Institute sequenced the *Bd* reference genome (JEL423) in 2007. The first population genomic study of *Bd* was published in 2011 with 29 sequenced isolates (Farrer *et al*. 2011). Incidentally, in 2011 the research that forms this dissertation commenced.

The of Bd life cycle

The *Bd* life cycle occurs over approximately 5 days in two main life stages: the zoospore stage, and the thallic, zoosporangium stage (Figure 1-1). The dispersal stage is a flagellated zoospore (Figure 1-1a), which can be motile for 12-24 hours, and can swim up to 2 cm before encysting (intracellularly; Greenspan *et al*. 2012) in host epithelial cells. Once encysted in a host cell, the zoospore develops into the spore-producing phase: the zoosporangium. Zoosporangia expand and fill with mitotically developing zoospores (Longcore 1999). Finally the new generation of zoospores is released through a discharge opening in the apex of the zoosporangium (the discharge papilla; Figure 1-1e).

It is noteworthy that all described stages of the *Bd* life cycle are thought to be functionally diploid (with high individual variation in chromosomal aneuploidy, ranging from 1-5 copies; Rosenblum *et al*. 2013, Farrer *et al*. 2013). The zoospores are clonally produced through mitosis, and no meiotic or haploid stage has yet been observed. It is unclear how mechanistically *Bd* lineages outcross to form hybrid strains like those described herein. One mechanism may be through a parasexual cycle, known from other fungi (Buxton 1965), where functional diploids fuse genomes without meiosis. The resulting multi-ploid genomes then might rapidly lose chromosome copies to produce the degree of variable aneuploidy observed across *Bd* genomes. Sexual reproduction is known from other members of Chytridiomycota (Barr 2001), but detailed studies on the subject are rare, and much remains unknown about reproductive evolution in most of this group.

Bd Population genetics and recent discoveries

When research for this dissertation started, all known genetic variation in *Bd* was restricted to a single rapidly expanding clonal lineage with a pattern of low genetic polymorphism without obvious geographic structure (James *et al.* 2009). In retrospect this was likely because the first, well-sampled population genetic studies focused their collection efforts on regions with the most conspicuous amphibian die offs (California: Morgan *et al*. 2007; Vredenburg *et al.* 2010; Central America: Lips *et al*. 2006; Cheng *et al*. 2011; and Australia: Berger *et al*. 1998). This global pandemic clone (the *Bd*-GPL, for *Global Panzootic Lineage*; Farrer *et al*. 2011) has now been detected in every genetically sampled chytridiomycosis outbreak causing die-offs and population collapse.

Our understanding of *Bd* genetic variation shifted dramatically in the period from 2011-2013. During this time several major studies revealed the existence of novel *Bd* genotypes deeply divergent from the globally invasive *Bd*-GPL (Farrer *et al*. 2011; Schloegel *et al*. 2012; Bataille *et al*. 2013). These newly discovered genotypes are described from geographic localities (Korea, South Africa, Switzerland, and Brazil) that have not typically experienced disease-associated amphibian declines, suggesting endemic pathogen populations in an equilibrium state and demonstrating that the evolutionary history of *Bd* is substantially more complex than previously realized. Schloegel *et al*. (2012) also reported a cross-strain hybrid between *Bd*-GPL and a novel endemic lineage in the Atlantic Forest region of Brazil. This was the first evidence of sexual reproduction in what was long thought to be a strict clonal pathogen. Finally in 2013, a congeneric sister species to *Bd* was discovered that specializes on attacking salamanders (*Batrachochytrium salamandrivorans*; Martel *et al*. 2013).

We now understand that the *Batrachochytrium* evolutionary tree is composed of multiple anciently diverged lineages and a sister species (Rosenblum *et al.* 2013; Martel *et al*. 2013), likely with more novel branches that have yet to be discovered. The current pace of discovery in *Batrachochytrium* biology is rapid at the current time, with new studies published almost routinely that disrupt our prior understanding of this organism's basic biology. Today, genomic sequencing is revolutionizing how we understand this pathogen. With recent discoveries though, come new questions and previous gaps in our knowledge revealed. Upon this background of understanding, this dissertation aims to clarify the population-level relationships between the newly discovered divergent strains of *Bd*, understand the genetics of reproduction in this species, and to understand the outcomes of population interactions between its deeply diverged lineages.

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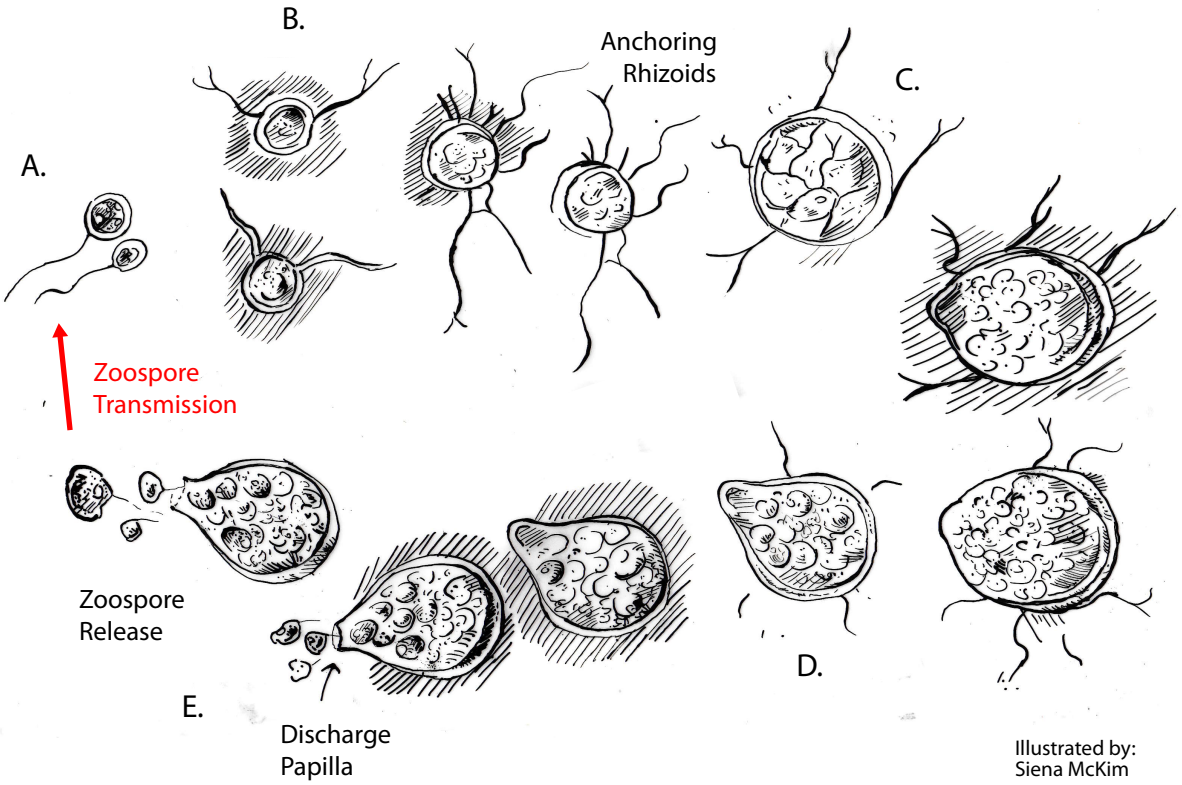

Figure 1-1: The life cycle of *Batrachochytrium dendrobatidis* (*Bd*). *Bd* is transmitted by aquatic, motile zoospores (A). Zoospores encyst within 12-24 hours of release, and encysted spores begin to develop anchoring rhizoids when grown in culture (B). New zoospores are produced mitotically in the growing zoosporangium (C). Maturing zoosporangia begin to form the discharge papilla (D). In approximately 5 days after encysting the discharge papilla open to release the next generation of mature zoospores. Illustrations are by Siena McKim, undergraduate art and design intern for the James Laboratory, University of Michigan, Penny W. Stamps School of Art and Design class of 2019. Observations of the Brazilian Atlantic Forest culture isolate CLFT035 were made with phase-contrast microscopy at 400x and 1000x magnification.

Chapter 2

Batrachochytrium dendrobatidis in Brazil comprises both locally endemic and globally expanding populations [1]

Abstract:

Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), is the emerging infectious disease implicated in recent population declines and extinctions of amphibian species worldwide. *Bd* strains from regions of disease-associated amphibian decline to date have all belonged to a single, hypervirulent clonal genotype (*Bd*-GPL). However, earlier studies in the Atlantic Forest of southeastern Brazil detected a novel, putatively enzootic lineage (*Bd*-Brazil), and indicated hybridization between *Bd*-GPL and *Bd*-Brazil. Here we characterize the spatial distribution and population history of these sympatric lineages in the Brazilian Atlantic Forest. To investigate the genetic structure of *Bd* in this region, we collected and genotyped *Bd* strains along a 2400 km transect of the Atlantic Forest. *Bd*-Brazil genotypes were restricted to a narrow geographic range in the southern Atlantic Forest, while *Bd*-GPL strains were widespread and largely geographically unstructured. *Bd* population genetics in this region support the hypothesis that the recently discovered Brazilian lineage is enzootic in the Atlantic Forest of Brazil, and that *Bd*-GPL is likely a more recently expanded invasive. We collected additional hybrid isolates that

[1] This chapter is published as Jenkinson, T. S., Betancourt Román, C. M., Lambertini, C., Aguilar-Valencia, A., Rodriguez, D., Nunes-de-Almeida, C. H. L., Ruggeri, J., Belasen, A. M., da Silva Leite, D., Zamudio, K. R., Longcore, J. E., Toledo, L. F. and James, T. Y. (2016). Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations. *Molecular Ecology* 25: 2978-2996.

demonstrate the recurrence of hybridization between panzootic and enzootic lineages, thereby confirming the existence of a hybrid zone in the Serra da Graciosa mountain range of Paraná State. Our field observations suggest that *Bd*-GPL may be more infective toward native Brazilian amphibians, and potentially more effective at dispersing across a fragmented landscape. We also provide further evidence of pathogen translocations mediated by the Brazilian ranaculture industry with implications for regulations and policies on global amphibian trade.

Introduction:

Novel fungal diseases are on the rise worldwide (Fisher *et al.* 2012). Highly destructive wildlife and human mycoses continue to emerge including: white nose syndrome of bats (*Pseudogymnoascus destructans*; Blehert *et al.* 2009; Gargas *et al*. 2009; Minnis & Lindner 2013), fungal meningitis (*Cryptococcus* species; Kidd *et al*. 2004; Bartlett *et al*. 2008), and valley fever (*Coccidioides* species; Kirkland & Fierer 1996; Burt *et al*. 1997; Fisher *et al*. 2000). Chytridiomycosis may be the most notorious of these emerging mycoses due to its contributions to dramatic amphibian declines worldwide and its potential to lead to massive biodiversity loss (Berger *et al.* 1998; Rachowicz *et al.* 2006; Skerratt *et al*. 2007). Although the current distributions of these mycoses are often well documented, the factors contributing to their emergence and spread remain largely unknown (Fisher *et al.* 2012). An accurate reconstruction of past disease expansion – including the timing and geography of emergence, as well as the selective environment underlying virulence evolution – is necessary if we are to successfully mitigate the emergence of these pathogens. Understanding pathogen geographic and genetic history is also critical to the prediction of future emergences, new host affiliations, and disease

outcomes under different environmental scenarios (Burt *et al.* 1996; Fisher *et al.* 2001; Wood *et al.* 2012).

Chytridiomycosis is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (hereafter *Bd*; Longcore *et al*. 1999), which now occurs on all continents except Antarctica (Olson *et al.* 2013). A number of recent studies have explored the genetics of *Bd* associated with amphibian communities in regions experiencing declines. In the best-studied regions of chytridiomycosis outbreaks (California: Morgan *et al*. 2007; Vredenburg *et al.* 2010; Central America: Lips *et al*. 2006; Cheng *et al*. 2011; the Pyrenees: Walker *et al*. 2010; and Australia: Berger *et al*. 1998; Murray *et al*. 2010), *Bd* has recently arrived and in some cases is still spreading. Outbreak-associated pathogen strains in these regions all belong to a single, rapidly expanding clonal lineage (James *et al.* 2009). This globally distributed clone, termed *Bd*-GPL (for *Global Panzootic Lineage*), shows a pattern of low genetic polymorphism without obvious geographic structure (Farrer *et al.* 2011). Recent surveys, however, have revealed the existence of novel *Bd* genotypes that are deeply divergent from potentially the hypervirulent *Bd*-GPL (Farrer *et al*. 2011; Schloegel *et al*. 2012; Bataille *et al*. 2013). These newly discovered genotypes are described from geographic localities (Korea, South Africa, Switzerland, and Brazil) that typically are not experiencing disease-associated amphibian declines, demonstrating that the evolutionary history of *Bd* is substantially more complex than previously realized. We now understand that the *Bd* evolutionary tree is composed of multiple anciently diverged lineages (Rosenblum *et al.* 2013), likely with more novel branches that have yet to be discovered.

Our study focuses on the Atlantic Forest (AF) of southeastern Brazil, where one recently discovered novel lineage, *Bd*-Brazil, is hypothesized to be enzootic (Schloegel *et al.* 2012). We chose to investigate the regional population genetics of *Bd* in this zone of deep ancestral variation because the pathogen dynamics in the AF remain enigmatic. *Bd* is widespread in southeastern Brazil (Toledo *et al.* 2006; Lisboa *et al.* 2013; Valencia-Aguilar *et al.* 2015), however the dramatic, rapid declines of amphibian species, well documented in other Neotropical regions (Lips *et al.* 2006; Cheng *et al.* 2011), have not been observed here. The few modern reports of amphibian declines and local extinctions in this area have not been directly attributed to the emergence of *Bd*, though their timing is contemporaneous with those in the rest of Latin America (Heyer *et al.* 1988; Eterovick *et al.* 2005; Silvano & Segalla 2005).

Retrospective studies of museum-preserved amphibians in Brazil suggest that *Bd* infection prevalence has remained constant in the coastal AF for over a century (approximately 24% prevalence since 1894; Rodriguez *et al*. 2014). Furthermore, highly divergent lineages (*Bd*-GPL and *Bd*-Brazil) that separated from a common ancestor up to 105,000 years ago coexist there (Rosenblum *et al.* 2013), and are capable of hybridizing (Schloegel *et al.* 2012). This is the first report of outcrossing in *Bd*, a pathogen initially thought to only reproduce asexually (Morehouse *et al.* 2003; James *et al.* 2009). Evidence that *Bd* is capable of a sexual cycle in this part of its range is of significant consequence to *Bd* pathogen dynamics, because this creates the possibility that the evolution of virulence in this region, and elsewhere, may be accelerated by sexual recombination.

The Brazilian AF is a major global biodiversity hotspot (Myers *et al.* 2000). Although the biome is highly fragmented and deforested with over 84% of its original range lost (Ribeiro *et al.* 2009), Brazil boasts the highest diversity of amphibian species of any nation (Wake & Vredenburg 2008), and around 60% of these amphibian species are endemic to the AF (Haddad *et al.* 2013). Brazil is also home to the greatest number of North American bullfrog (*Lithobates catesbeianus*) farms in the Western Hemisphere (Schloegel *et al.* 2010). Bullfrogs are highly tolerant to *Bd* infection, show limited disease symptoms (Garner *et al.* 2006), and have become established throughout southeastern Brazil (Both *et al.* 2011), making them a potential vector species (Rödder *et al.* 2013). The ranaculture export industry in Brazil introduces the additional dynamic of non-native amphibians with the capacity to transmit *Bd* asymptomatically, and presents a plausible mechanism for the inter-continental movement of *Bd* genotypes (James *et al.* 2015).

Here we report on a large-scale regional sampling of field-isolated *Bd* strains from the Brazilian AF, with the goal of characterizing the spatial distribution and population genetic structure of Brazilian *Bd* lineages relative to the globally distributed *Bd*-GPL. These sympatric populations of divergent lineages are an excellent system with which to explore the roles of genetic structure, sexual recombination, and local adaptation in shaping the evolution of hypervirulent pathogens. Specifically, our aims were to elucidate the geographic distribution of divergent *Bd* genotypes across the AF, to quantify genetic diversity within and among *Bd* populations occurring in Brazil, to determine whether strains are long-term enzootics or recently introduced, and to identify the extent of sexual recombination and hybridization in the region. We also assessed the relationship of *Bd* genotypes recovered from the AF to a global pool of

previously described *Bd* strains. Combined, our results provide insight into the history of chytridiomycosis in a crucial region of amphibian biodiversity and relate the genetics of *Bd* in this region with that of the ongoing global panzootic.

Materials and Methods:

Field Sampling and Pathogen Isolation

During peak rainfall months (January through February) of 2013 and 2014, we collected native larval anurans at ten collection sites across six Brazilian states. The infection patterns in amphibian larvae provide a reasonable proxy for infection patterns in the amphibian community across developmental stages. Larvae have been shown to maintain infection throughout metamorphosis (McMahon & Rohr 2015) and are readily infected with *Bd* strains carried by adults sharing the same environment (Greenspan et al. 2012; Bataille et al. 2013) as most amphibian species do in the Brazilian AF (Haddad *et al.* 2013). Our north-south transect spanned 2400 km of the AF from the northeastern state of Bahia to the southeastern state of Santa Catarina (39.55°W, 15.42°S to 49.9°W, 27.67°S; Figure 2-1). We represented collection points less than 10 km apart by a central coordinate for geographic analyses.

We used a 10X hand lens to screen larvae in the field for signs of chytridiomycosis by assessing the level of oral tissue dekeratinization (Knapp & Morgan 2006). We euthanized animals with signs of *Bd* infection by pithing the brain and spinal cord immediately before confirming the infection with a compound microscope. We dissected infected oral tissues for pathogen isolation on 1% tryptone agar with 0.2 mg/mL penicillin-G and 0.4 mg/mL streptomycin sulfate (Longcore 2000). Isolates of *Bd* were maintained on 1% tryptone agar at 20-

21º C until sufficient growth had occurred for DNA extraction. Finally, we cryopreserved replicate cultures of all isolates at -80 °C in 1% tryptone broth with cryoprotectant solution (Boyle *et al.* 2003) and deposited them in the University of Maine chytrid culture collection (JEL) and the Universidade Estadual de Campinas *Bd* culture collection (CLFT).

Multilocus Sequence Typing

Due to increasing awareness that the genomes of *Bd* isolates change through prolonged laboratory culture (Langhammer et al. 2013; Voyles et al. 2014), we only passaged new isolates two to three times as necessary before DNA extraction. We harvested mature zoospores and sporangia from ~ 7 day old culture transfers by aseptically scraping fungal tissue from the surface of the agar medium. We used a standard CTAB miniprep protocol with chloroform and isoamyl alcohol to extract DNA from *Bd* isolates (Zolan & Pukkila 1986). We then amplified DNA extracts with ExTaq DNA polymerase (TaKaRa), and purified the PCR products using ExoSAP-IT (Affymetrix). We Sanger sequenced 12 polymorphic multilocus sequence typing (MLST) loci on an ABI 3730 DNA analyzer (Applied Biosystems) at the University of Michigan DNA Sequencing Core. Seven of these MLST markers were previously described (*8009X2*, *BDC5*, *BdSC3.1*, *BdSC4.16*, *BdSC6.15*, *BdSC7.6*, *R6064*; Morehouse *et al*. 2003; Morgan *et al*. 2007; James *et al*. 2009; Schloegel *et al*. 2012). Because previously published markers were designed before the discovery of the *Bd*-Brazil lineage, and may be biased toward capturing variation in *Bd*-GPL, we designed five new markers for this study. (*BdSC2.0*, *BdSC6.8*, *BdSC9.1*, *BdSC11.5*, *BdSc16.2*; Table 2-1). For a subset of our samples, we also sequenced markers *BDC24* (James *et al.* 2009), *BdSC4.3*, and *BdSC8.10* (Schloegel *et al.* 2012) to compare

with previously published global *Bd* genotypes; however, we discontinued sequencing of these markers when they were observed to be monomorphic within each major lineage in our transect.

To develop the additional markers, we explored a data set of published *Bd* genomes, including representatives of the *Bd*-GPL, *Bd*-Cape and *Bd*-Brazil lineages (Farrer *et al.* 2011; Rosenblum *et al.* 2013), and searched for regions of high potential heterozygosity with a custom-designed, sliding-window PERL script. We also found protein-coding regions containing trinucleotide repeat expansions, which are known to be of potential utility as population informative markers (Di Rienzo *et al.* 1994; Orr & Zoghbi 2007), by BLASTN of the reference genome of *Bd* (JEL423; Broad Institute version 17-Jan-2007). We then screened regions of high relative heterozygosity and variable repeating sequence for polymorphic sites by designing primers in flanking regions using PRIMER 3 (Rozen & Skaletsky 1999).

Data Analyses

We assigned genotypes to each *Bd* isolate by comparing nucleotide sequences to reference sequences with SEQUENCHER v4.10.1 (GeneCodes). We calculated descriptive indices of molecular diversity including observed heterozygosity (H_O), and average gene diversity (expected heterozygosity, H_E; Nei 1987) with ARLEQUIN v3.5.1.3 (Excoffier & Lischer 2010). To quantify the degree of genetic similarity between geographic populations, we calculated pairwise F_{ST} values between populations with ARLEQUIN and constructed a population level neighbor-joining dendrogram from the resulting F_{ST} matrix with the R package GPLOTS.

In the absence of sexual reproduction, clonal diploid lineages are predicted to accumulate

heterozygosity through mutation leading to highly negative F_{IS} values (De Meeûs *et al*. 2006). To test for evidence of historical recombination within lineages, we calculated global and locus specific F_{IS} values for individual populations using Weir and Cockerham's (1984) method implemented in GENEPOP v4.0.10 (Rousset 2008). We also conducted Hardy-Weinberg (HW) exact tests for deviations from expectation under a random mating model for each locus with GENEPOP. As an alternative test for recombination utilizing disequilibrium among loci, we determined the index of association (I_A; Smith *et al.* 1993; Agapow & Burt 2001) for each geographic population. The index of association (I_A) describes the degree of disequilibrium between genotypes, and has been useful in inferring the occurrence of cryptic recombination in putatively asexual populations (Burt *et al.* 1996). We tested for significant deviation from 1000 random multilocus permutations of genotypes under a random mating model with POPPR v1.1.2 (Kamvar *et al.* 2014).

We used PAUP* v4.0b10 (Swofford 2002) to construct a neighbor-joining dendrogram of newly collected isolates and previously published genotypes after clone-correction (removal of identical genotypes within a geographical population to account for non-independent sampling). We estimated genetic distance between genotypes for this analysis with a *hetequal* coding strategy, which assumes heterozygous polymorphisms in each marker to be one step from the nearest heterozygote and two steps from other heterozygotes (Mountain & Cavalli-Sforza 1997; James *et al.* 2009). Support values for clades in the neighbor-joining dendrogram were inferred by bootstrapping over 1000 replicates. We visualized genotype clustering of our samples within a globally sampled panel of previously published *Bd* genotypes with a principal components analysis (PCA) conducted using R packages ADE4 (Dray & Dufour 2007) and ADEGENET

(Jombart 2008). For this analysis, we were constrained to a set of markers overlapping with those sequenced in prior studies. Because of this, we used a subset of our isolates for which the monomorphic markers *BDC24*, *BdSC4.3*, and *BdSC8.10* were sequenced. Finally, we constructed a summary map of genotype distributions in southeastern Brazil using the R packages MAPTOOLS and PLOTRIX.

Ethics statement

We performed all investigations involving live animals and the international export of pathogen cultures following protocols approved by the University of Michigan's Institutional Animal Care and Use Committee (protocols: PRO00000009 and PRO00005605), and the Brazilian Ministry of the Environment's Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio permits: 27745-8 and 35779-4).

Results:

Heterogeneous distribution of enzootic and hybrid lineages

We successfully isolated 111 new strains of *Bd* from infected anurans across our sampling transect (Table A-1) and analyzed them along with eleven previously published Brazilian isolates, including five isolates from Brazilian farmed *L. catesbeianus* (Schloegel *et al.* 2012). We recovered 77 unique multilocus genotypes (MLG) after clone-correcting our dataset; 61 were *Bd*-GPL and 14 were *Bd*-Brazil (Table A-2). We collected two new hybrid strains represented by a single clonal MLG, which was distinct from that of the hybrid strain originally reported by Schloegel *et al.* (2012). For our seven lineage-informative markers (*8009X2*, *BDC5*, *BdSC2.0*, *BdSC4.16*, *BdSC6.15*, *BdSC6.8*, *BdSC9.1*), there were no shared alleles between the

Bd-GPL and *Bd*-Brazil lineages. Our hybrid strains were always heterozygous with one allele from each parental lineage at each of these informative markers. Additionally, no more than two alleles were ever observed at any of the 12 markers, confirming that these were hybrid strains, and not cases of coinfection by *Bd*-Brazil and *Bd*-GPL.

Bd-Brazil and hybrid genotypes were confined to a narrow coastal zone between 23°S and 27°S in the southeastern AF (Figure 2-1). Representatives of the globally distributed *Bd*-GPL lineage were found at all ten of our sampling sites and were the only genotypes present on non-native amphibians (Table A-1). The PCA with a global pool of published *Bd* genotypes showed that the Brazilian AF harbors a high level of overall genetic diversity when compared to the global panel of *Bd*-GPL strains (Figure 2-2). The diagnostic marker *R6046* (Morehouse *et al.* 2003), which differentiates the mostly temperate North American/European *Bd*-GPL-1 clade of Schloegel *et al.* (2012) from the globally distributed *Bd*-GPL-2, showed that all of our *Bd*-GPL representatives belonged to the globally distributed *Bd*-GPL-2 group except for two isolates from Reserva Betary in São Paulo State which belonged to *Bd*-GPL-1 (Figure 2-3).

The proportion of enzootic and hybrid genotypes across all sampled sites in our transect was 23.9% (21.4% *Bd*-Brazil; 2.5% hybrids). However, the prevalence of non *Bd*-GPL genotypes, in sites where present, ranged from 80.0% (8/10) in Serra do Japi, São Paulo; and 73.1% (19/26) in Serra da Graciosa, Paraná; to 20.0% (1/5) in Pomerode, Santa Catarina. *Bd*-Brazil and hybrid genotypes were not found at the northern or southern extremes of the transect. Where present, these genotypes were restricted to hosts in the genera *Hylodes* and *Bokermannohyla*. Our two newly isolated hybrid strains were from *Bokermannohyla hylax* hosts,

both from the Serra da Graciosa hybrid site in the state of Paraná where a previous hybrid strain was reported (Schloegel *et al.* 2012).

Patterns of genetic diversity of Bd *lineages in the Atlantic Forest*

Global heterozygosity (H_O) across all AF isolates was 0.473, gene diversity (H_E) was 0.511 (Table 2-2), and the inbreeding coefficient (F_{IS}) was 0.074 after clone-correction. When analyzed independently, all major lineages had negative F_{IS} values, indicating an excess of heterozygotes relative to HW equilibrium expectations (Table 2-3). The *Bd*-GPL lineage had slightly higher overall H_O across all alleles compared to the global mean (0.475), while *Bd*-Brazil was slightly less heterozygous (0.423); but this difference in heterozygosity was not significant (Wilcoxon rank-sum test, $P = 0.885$). As expected, the hybrid isolates displayed significantly higher levels of observed heterozygosity than the other lineages ($H_O = 0.750$; Wilcoxon rank-sum test, $P = 0.012$).

Mean gene diversity (H_E) across populations differed significantly between lineages, 0.374 in *Bd*-GPL and 0.287 in *Bd*-Brazil (Wilcoxon rank-sum test, $P = 0.041$). Evidence of marker ascertainment bias was observed, however, when our newly developed markers were analyzed separately. The significant difference in average gene diversity was not evident when mean H_E was calculated using only our new markers designed from genome sequences of *Bd*-Brazil (Figure 2-4; Wilcoxon rank-sum test, $P = 0.909$), whereas previously published markers analyzed separately differed in mean H_E (Wilcoxon rank-sum test, $P = 0.030$). Average allele richness over all loci ranged from 1.725 alleles in *Bd*-GPL to 1.667 in *Bd*-Brazil. The hybrid population had significantly higher gene diversity than the other lineages ($H_E = 0.569$; Wilcoxon

rank-sum test, $P = 0.004$), and elevated mean allele richness (2.083 alleles). Genotypic diversity (defined as the proportion of unique MLGs per sample) of the entire AF dataset was 0.658. Genotypic diversity did not differ between lineages, with average genotypic diversities of 0.583 in *Bd*-Brazil versus 0.685 in *Bd*-GPL (Wilcoxon rank-sum test, $P = 0.731$). One of our twelve sampled loci (*BdSC16.2*) was monomorphic in *Bd*-GPL, whereas three loci (*8009X2*, *BDC5*, *BdSC4.16*) were monomorphic in *Bd*-Brazil.

Population genetic structure of Atlantic Forest Bd *isolates*

Both lineages were subdivided by geography (Fisher's exact test; both lineages: $P < 0.001$). A clustering dendrogram constructed from pairwise F_{ST} values between *Bd*-GPL populations with more than three sequenced isolates grouped geographic populations into two major groups with high bootstrap support (Figure 2-5). These groups are unexpectedly structured in that three populations from the extreme northern transect (Serra Bonita, Bahia; Vargem Alta, Espírito Santo; and Santa Teresa, Espírito Santo) cluster with the extreme southern population of Rancho Queimado, Santa Catarina. Geographic subpopulations of *Bd*-GPL were weakly isolated by distance ($r = 0.012$; Mantel test $P = 0.037$). We did not test for significant isolation by distance in *Bd*-Brazil populations due to the limited sample size of populations.

Despite the significant subdivision among populations, four *Bd*-GPL MLGs were shared among sample sites in our transect indicating gene flow, or recent, rapid expansion (Figure 2-1 and Figure 2-3). The population of Serra Bonita, Bahia shared one MLG each with the adjacent northeastern sample sites of Santa Teresa and Vargem Alta, both in Espírito Santo State (maximum distance = 521 km). Serra dos Órgãos, Rio de Janeiro State and Bertioga in São Paulo

State (distance = 342 km) shared one MLG. The greatest distance between shared MLGs from native amphibians was between Serra Bonita and Serra dos Órgãos (891 km). We also found shared MLGs associated with the ranaculture industry. Isolates from one bullfrog farm in Tremembé, São Paulo shared MLGs with those from both native and farmed amphibians. This bullfrog farm isolate (LMS931) shared a clonal genotype with an isolate collected in Serra dos Órgãos, a protected national park. The Tremembé farm isolate also shared a genotype with an isolate from a bullfrog farm in Belém, Pará (Schloegel *et al.* 2012), in the Amazon River delta separated by over 2600 km. No shared *Bd*-GPL MLGs were observed in the southwestern sample sites of the collection transect, and no MLGs were shared between *Bd*-Brazil populations (maximum distance = 320 km).

The neighbor-joining dendrogram (Figure 2-3) revealed a lack of geographic structure in the *Bd*-GPL lineage. Instead, clades were composed of isolates from disparate geographic populations and several clades included *Bd*-GPL populations from extremes of the AF transect. Conversely, geographic populations of *Bd*-Brazil form site-specific clades with the exception of the isolate CLFT071 from Serra do Japi, São Paulo State, which forms a clade with the *Bd*-Brazil isolate UM142, originally cultured from a captive *L. catesbeianus* for sale in a United States food market (Schloegel *et al.* 2012). The PCA of AF isolates with a global pool of previously sequenced isolates showed significant clusters representing all genotypic lineages known to occur in the Western Hemisphere (Figure 2-2), with a total of 21.7% of genetic variation explained by the first three principal components. Brazilian AF MLGs of *Bd* are represented in each cluster. The PCA also shows the *Bd*-GPL clade forming two clusters representing the *Bd*-GPL-1 and *Bd*-GPL-2 split. Our AF *Bd*-GPL-1 representative, for which we sequenced sufficient

overlapping loci with previously sequenced isolates, was separated from the rest of the *Bd*-GPL-1 cluster. The two Brazilian hybrid MLGs are separated across all three axes of our PCA indicating an appreciable degree of genetic distance between hybrid MLGs.

Signatures of recombination in Atlantic Forest Bd *populations*

Both *Bd*-GPL and *Bd*-Brazil lineages had highly negative F_{IS} estimates, as predicted for a predominantly asexual population (De Meeûs *et al*. 2006). We calculated F_{IS} values from clone-corrected data to control for non-independent clonal samples (Table 2-3). The *Bd*-GPL displayed an F_{IS} closer to zero (-0.245) than the *Bd*-Brazil lineage (-0.416). Both the *Bd*-GPL and *Bd*-Brazil lineages deviated from expected heterozygosities under HW equilibrium expectations ($P < 0.001$ and $P = 0.0012$, respectively). However, not all loci matched these trends. When analyzed by lineage using HW exact tests, we failed to reject the null expectation for 36.4% (4/11) of the informative markers in the *Bd*-GPL group (significance cutoff $\alpha = 0.05$). Within the *Bd*-Brazil lineage, we failed to reject the null expectation in 42.9% (3/7) of the informative markers. To eliminate the potential artifact of reduced heterozygosity in pooled populations that are significantly subdivided (the Wahlund effect), we also performed HW exact tests on each geographic population with more than three sequenced MLGs. Among the nine *Bd*-GPL populations with adequate sampling, 81.3% (61/75) of the informative markers did not differ from null HW expectations, and 76.9% (10/13) of the informative loci did not significantly differ from null expectations in the *Bd*-Brazil populations.

In a separate test for historical recombination, genotype data from both the *Bd*-GPL and *Bd*-Brazil lineages were randomly shuffled over 1000 permutations using a non-parametric

bootstrap resampling approach to generate a null distribution of I_A valuess under a random recombination model. The observed index of association estimated for *Bd*-GPL significantly differed from the randomized distribution (Table 2-3 and Figure 2-6a; $P = 0.009$), whereas the I_A of our *Bd*-Brazil dataset did not (Figure 2-6b; $P = 0.465$).

Discussion:

Emerging fungal pathogens are a growing threat to global biodiversity, and have already disrupted host populations throughout a range of habitats (Fisher *et al*. 2012). Despite the urgent need to comprehend the causes and consequences of disease emergence, our understanding of fungal pathogen biology lags behind that of other taxonomic groups (Giraud *et al*. 2008), which in turn hinders an informed response to their outbreaks. Prior population studies of fungal pathogen systems have revealed that divergent host adaptation (Fisher *et al*. 2005; Gladieux *et al*. 2011), recombination (Stukenbrock *et al*. 2012), and pathogen translocation to new environments (Gladieux *et al*. 2015) may all play important roles in emergence. Our study presents a large-scale regional sample of genotyped *Bd* isolates from the Brazilian AF. The AF is the only global region where all of the aforementioned forces appear to have contributed to local *Bd* population dynamics. As such, the examination of these populations provides a valuable opportunity to better understand the evolutionary history of *Bd*, and to predict the consequences of lineage divergence, hybridization, and strain translocation on disease outcomes as chytridiomycosis continues to spread to new environments.

Long-term population history of Bd *in the Atlantic Forest*

The only extensive prior study of *Bd* in the Brazilian AF focused on a temporal sampling of museum-preserved amphibian specimens dating back to 1894 (Rodriguez *et al*. 2014). In that study, the authors genotyped 52 *Bd* infections from skin swabs using a single ribosomal marker (ITS1), and concluded that *Bd* had not been introduced to Brazil over their 116 year sampling period. Based on those results, the authors hypothesized that both the *Bd*-GPL and *Bd*-Brazil lineages may have been endemic to the AF. However, based on a single hyper-variable marker (Nilsson *et al*. 2008; Bataille *et al*. 2013), the Rodriguez *et al*. (2014) study was not able to address the history of *Bd* in Brazil before their earliest sample. On the other hand, our multilocus dataset provided a more robust opportunity to make inferences about population history before 1894. Both of our studies conclude that *Bd*-Brazil is an endemic lineage to the AF, but our study calls into question the hypothesis that *Bd*-GPL originated in Brazil. The combined evidence between our two studies agree that *Bd*-GPL was already in Brazil before the import, and subsequent escape, of the North American bullfrog for trade in the early 20th century, but the question to be resolved is whether *Bd*-GPL has been present in the AF as a long-term endemic.

Population genetic theory predicts lineages that have been stable in a given locality should have proportionally greater genetic diversity than recently translocated lineages due to the founder effect (Hartl & Clark 1997). Based on our analyzed set of marker loci, it would initially appear that *Bd*-GPL is as genetically diverse as *Bd*-Brazil. Upon further investigation, however, multiple lines of evidence from our study support the hypothesis that the *Bd*-Brazil lineage may have been present in the Brazilian AF longer than *Bd*-GPL.

First, estimates of genetic diversity based on population markers designed before the discovery of novel Brazilian lineages are confounded by an inherent bias toward capturing variation in *Bd*-GPL and not in *Bd*-Brazil. When our newly developed markers are analyzed independently, a difference in gene diversity (H_E) between the two lineages is no longer observed (Figure 2-4). In a recent study of comparative genomic diversity which included two *Bd*-Brazil isolates and a global panel of *Bd*-GPL isolates (Rosenblum *et al*. 2013), higher heterozygosity was observed within *Bd*-Brazil strains lending support to our hypothesis at the genomic level. Within our dataset, other historical factors specific to Brazil may have also had an effect on current day diversity estimates. Multiple successive introductions of *Bd*-GPL – which we infer must have occurred at least twice based on the co-occurrence of both major GPL genotypes (*Bd*-GPL1 and *Bd*-GPL2) in the Reserva Betary population – would increase diversity in the *Bd*-GPL obscuring the expected differences in diversity between the *Bd*-GPL and *Bd*-Brazil lineages. Because of these variable factors influencing our observed genetic diversity, we chose not to base our conclusions on this line of evidence, opting instead for stronger infra-lineage based comparisons.

Second, if *Bd*-GPL had been present as a long-term endemic in the AF, geographic structuring should be evident, especially after more than four centuries of anthropogenic habitat fragmentation introducing barriers to dispersal. Three geographical analyses independently show that *Bd*-GPL has not been present in the AF long enough for the establishment of geographic structuring. In contrast, *Bd*-Brazil is geographically structured, most likely as a result of long-term endemism. Our genotype dendrogram (Figure 2-3) shows a distinct lack of geographic structure in the *Bd*-GPL clade, whereas our *Bd*-Brazil genotypes form clades corresponding with

geographic origin. Likewise, when we cluster our *Bd*-GPL populations by pairwise F_{ST}, we observe only a minor relationship between genetic divergence and geography (Figure 2-5). The pairwise F_{ST} analysis indicates that geographically distant populations of *Bd*-GPL are often less differentiated from one another than they are to their adjacent counterparts, suggesting a rapid and recent expansion (Excoffier *et al.* 2009). While the possibility exists that historical geographic structure in *Bd*-GPL could be masked by recent long-range movement of *Bd*-GPL through the bullfrog trade, this scenario is unlikely given the lack of such long-range movement in *Bd*-Brazil, which is also know to infect bullfrogs in the ranaculture industry (Schloegel *et al.* 2012), and whose range overlaps with the potential invasive range of bullfrogs in the AF. It is unlikely that bullfrogs would differentially transmit *Bd*-GPL to produce the pattern we observe.

Third, only *Bd*-GPL populations share MLG clones, likely due to a recent spread of *Bd*-GPL. Even at short geographic distances, MLGs were never shared among *Bd*-Brazil populations, suggesting that these populations have been separated for longer periods of time without migration. Our observation of shared MLGs concentrated to northern *Bd*-GPL populations indicates that this lineage may have recently expanded northward. If, as we suspect, this pattern were produced by rapid expansion of a recently introduced *Bd*-GPL founding population, *Bd*-GPL populations should show little isolation by distance. Indeed, a Mantel test resulted in a weak correlation between genetic dissimilarity and geographic distance. Together, these analyses imply a scenario of *Bd*-GPL introduction within the last few centuries and reflect a relatively short period of time for the accumulation of variation between populations. Again, it is difficult to discern between our hypothesis of historical expansion and a recent increase in gene flow between current populations as the cause of this pattern. Given the highly fragmented

nature of the AF, we believe that the former scenario is more plausible. The significant isolation by distance we observe between *Bd*-GPL populations, albeit weak, indicates that any recent gene flow between populations would have been minimal. We cannot, however, discount the possibility that anthropogenic movement of amphibians may have played a role in shaping the population structure of *Bd*-GPL in these native amphibian hosts.

Finally, differences in the significant association of alleles from the randomly permutated datasets may indicate major differences in the population histories of the two divergent AF lineages (Figure 2-6). Under random recombination over sufficient time, the index of association between alleles in a population is predicted to approach zero (Smith *et al.* 1993). In clonal populations – where recombination has been rare or absent – alleles are passed on to asexual daughters in complete disequilibrium, resulting in significantly non-zero I_A values as seen in *Bd*-GPL populations. In contrast, our results indicated that the *Bd*-Brazil lineage has been present in the AF long enough to display genotypic equilibrium through rare recombination. The same tests repeated within our subdivided populations show that the significant association of alleles in the *Bd*-GPL is not solely due to population subdivision. There may be several possible explanations for the disparity in the association of alleles between lineages. One possibility is that recombination rates differ between the two lineages. A study of the recently discovered, divergent Swiss (*Bd*-CH) and African (*Bd*-Cape) lineages suggested that representatives of the divergent lineages might have elevated rates of mitotic recombination relative to *Bd*-GPL (Farrer *et al*. 2013). Another possibility may be that the observed index of association in *Bd*-Brazil is likely a product of long-term demographic stability. Differential rates of recombination between

Bd-GPL and *Bd*-Brazil have never been examined, and while out of the scope of this study, should be a priority for future research.

Implications of current lineage distributions

One of the most striking aspects of our field data was the restriction of enzootic lineages (*Bd*-Brazil and hybrids) to a narrow portion of the AF. One explanation for this pattern may be that enzootic lineages require a higher degree of environmental or host specificity than the *Bd*-GPL lineage. Temperature and humidity are probable abiotic factors restricting the spread of enzootic lineages through the AF given that the latitudinal range in which we found *Bd*-GPL is much greater than that of *Bd*-Brazil. Whether *Bd*-GPL populations are better able to tolerate extremes in temperature and moisture, however, remains to be tested experimentally. Our results indicate that *Bd*-GPL arrived more recently to the AF than *Bd*-Brazil, and that it shows signatures of a recent demographic expansion. Taken together, these findings support the hypothesis that *Bd*-GPL may be a better disperser across fragmented landscapes. The southern range of the AF in the states of São Paulo, Paraná, and Santa Catarina contain the most intact remnant patches of forested terrain in coastal Brazil, whereas the northern transect in our study has experienced a history of greater deforestation (Pinto *et al.* 2014). Studies in this region have shown that *Bd* infection is more prevalent in pristine versus disturbed habitats (Becker & Zamudio 2011). A fruitful avenue for future research will be to determine whether certain *Bd* strains themselves are better able to tolerate extreme or degraded habitats, or whether they are better able to disperse through other mechanisms such as infective differences on specific host species.

Our sampling effort was not designed to explicitly address the question of differences in host specificity between lineages, but the predominant trend in our results is that *Bd*-GPL is able to infect a wider assemblage of amphibian hosts in the AF (Table A-1). Interestingly, the northernmost extent of *Bd*-Brazil's observed range coincides with a known biogeographic delimitation between northern and southern climatically adapted AF species (Carnaval *et al.* 2014). Taxonomic groups across this north/south split include many amphibians that may have diversified in separate biogeographical refugia (Carnaval *et al.* 2009; Thomé *et al.* 2010). Paleoclimatic modeling suggests that during the Late Quaternary glacial maxima, the AF was restricted to smaller, climatically stable refugia.

The predicted refugia most relevant to our collection transect are the large northern Bahia refugia, and a series of smaller southern refugia in the coastal regions of the present day states of São Paulo and Paraná. These refugia are centers of high host phylogenetic endemism (Carnaval & Moritz 2008), and the *Bd*-Brazil lineage has only been found within the southern center of historical diversification corresponding to the São Paulo and Paraná refugia. The geographic restriction of *Bd*-Brazil to this center of AF microendemism, in conjunction with our data supporting the long term endemism of this lineage, leads us to hypothesize that *Bd*-Brazil was similarly restricted to these southern refugia, where it became locally adapted to co-occurring southern host species. Subsequently, its current distribution may reflect a history of tracking hosts that remained confined to the southern AF due to a combination of habitat heterogeneity and migration barriers.

Sexual reproduction and a pathogen hybrid zone

Hybridization can be a driving force in the evolution of fungal pathogen populations (Stukenbrock *et al.* 2012). Studies of other eukaryotic pathogens show that major changes in phenotype by sexual recombination and hybridization can play a pivotal role in the emergence of virulence (Grigg *et al.* 2001; Sibley & Ajioka 2008). *Bd* genotypes that have been geographically or environmentally isolated should have diverged from each other over time as they adapted to local host defenses. Sexual outcrossing adds a new dimension by which *Bd* might explore the fitness landscape, particularly through the generation of variation in pathogenic phenotype. Experimental infections show that the original Brazilian hybrid strain CLFT024/02 causes greater mortality in a non-Brazilian amphibian host (*Lithobates sylvaticus*) than representative strains from either the parent *Bd*-Brazil or *Bd*-GPL lineages (Betancourt Román *et al*. in review). If similar effects occur in local host populations, the ecological implications could be serious, and the need for more robust biosecurity measures to prevent the export of hybrid strains from Brazil will be pressing.

Our survey recovered two new isolates of hybrid genotypes from Serra da Graciosa, the hybrid locality originally reported by Schloegel *et al.* (2012). Although sexual reproduction has not been directly observed in *Bd in vitro*, sexual recombination has likely been an important influence on its genetic history (James *et al.* 2009; Rosenblum *et al.* 2013). Our two new hybrid isolates appear to be genetic clones of each other (a single MLG), but significant genetic differences exist between our hybrid isolates and the originally described hybrid CLFT024/02. The hybrid MLGs are distinctly separated across all three axes in our PCA (Figure 2-2), and differ at five of our twelve sequenced markers. Of these differences, four loci show patterns inconsistent with the inheritance of alleles from the same *Bd*-GPL or *Bd*-Brazil gamete (*i.e.*,

different lineage specific parental alleles are present in either hybrid MLG). Hence these data demonstrate the occurrence of multiple hybridization events in the Paraná hybrid zone.

An alternative explanation is that these hybrid genotypes are divergent lineages resulting from a parasexual mating (a non-meiotic fusion of diploid parents with the subsequent loss of chromosomes back to the diploid state), which is known to occur in many groups of fungi (Buxton 1956; Caten & Jinks 1966). This would involve tetraploid intermediates and may explain the higher ploidy levels observed in CLFT024/02 (Schloegel *et al.* 2012). If hybridization was unrestricted, the expected frequency of hybrid strains should roughly equal the frequency of parental genotypes. Hybrid isolates are rarer than expected in the hybrid zone, which may be due to the incipient accumulation of Dobzhansky-Muller incompatibilities hindering the viability of hybrid offspring, or it could represent the rareness of mating opportunities. It remains to be determined whether specific ecological conditions in the Serra da Graciosa site promote the outcrossing of otherwise reproductively isolated lineages. This site may be a recent contact zone between two previously isolated mating types of *Bd* that recently came back into contact without having lost the ability to outcross.

When testing for the signature of historical sexual reproduction, we could not reject that genotypes in AF *Bd* populations were in HW equilibrium. Our analyses may have been constrained by sample size and the technical challenges involved in producing statistically powerful MLST data, but independent tests produce results inconsistent with a scenario of strict asexuality in both the *Bd*-Brazil and *Bd*-GPL lineages. Contrasting the results of our HW exact tests with our I_A permutation tests (which are more sensitive to rare recombination) indicates that

there is variance among loci in heterozygosity excess, a pattern that can be explained by very rare sex (Balloux *et al.* 2003) or by mitotic recombination with variable effects across loci. Genotypic equilibrium in *Bd*-Brazil is consistent with an older lineage, in which more time has allowed recombination to break down linkage associations. Furthermore, absence of HW equilibrium may show that some loci are under selection to maintain heterozygosity, perhaps through overdominance. Deeper knowledge about the historical degree of sexual reproduction in *Bd* may hold the key to the origin of the global chytridiomycosis panzootic. Our MLST data may not be sufficient to provide satisfactory conclusions about historical recombination events, because those genetic signatures may be eroded by mitotic recombination. Alternatively, a genome resequencing approach combined with predictive population genetic models of genomic heterozygosity under differing reproductive scenarios of may provide greater utility in addressing the influence of historical sexual recombination in shaping present day lineages of *Bd*.

Roles of anthropogenic disease translocation

Our results also provide evidence of recent genotype translocation between *Bd*-GPL populations in the northeast region of our sampling transect and the South American ranaculture industry. The incorporation of five strains recovered from captive *L. catesbeianus* at three Brazilian bullfrog farms and one United States food market (Schloegel *et al.* 2012) provide further insight into the role of the amphibian trade in the long-distance dispersal of *Bd* strains. Most revealing was the distance between shared MLGs (based on our 12 marker dataset) recovered from two geographically distant farms 2600 Km apart (in São Paulo State and Pará State). This is over three times the distance of shared MLGs between natural populations of

native amphibians in the AF, and shows that ranaculture in Brazil is responsible for long-distance *Bd* transmission. The *Bd*-Brazil representative previously isolated from a market in the Detroit metro area, Michigan, United States (UM142; Schloegel *et al*. 2012) forms a clade with a *Bd*-Brazil isolate from Serra do Japi, São Paulo (Figure 2-3). These results, along with the demonstrated niche overlap between *Bd* and *L. catesbeianus* (Roedder *et al.* 2013), illustrate the growing problem of pathogen transport through the South American bullfrog trade.

Conclusion

We hypothesize that the divergent *Bd* lineages in Brazil have each experienced very distinct population histories, but have been brought into close contact in portions of the AF. Our findings that *Bd*-Brazil has a higher degree of geographic structure and may have experienced a greater degree of historical recombination than *Bd*-GPL support a hypothesis of long-term endemism of *Bd*-Brazil, and one or more recent introductions, followed by rapid northward expansion of *Bd*-GPL. Our study expands the known range of the recently discovered *Bd*-Brazil lineage in the AF of Brazil, and we document the existence of a hybrid zone in the state of Paraná with the collection of additional hybrid isolates.

A better understanding of how genetic diversity and phenotypic differences in heterogeneous environments underlie selection on pathogen virulence will be necessary to predict and prevent future emerging diseases like chytridiomycosis. We suggest that crucial insights may be found by disentangling the interplay between cross-strain interactions such as competition and sexual recombination. Although we still have much to learn about these interactions between the pathogen lineages detailed herein, the population genetics of *Bd* in the

Brazilian AF show that both forces are probably shaping disease dynamics of the region, and that the long-range transport of these *Bd* genotypes are likely to pose consequences to pathogen evolution at the global scale.

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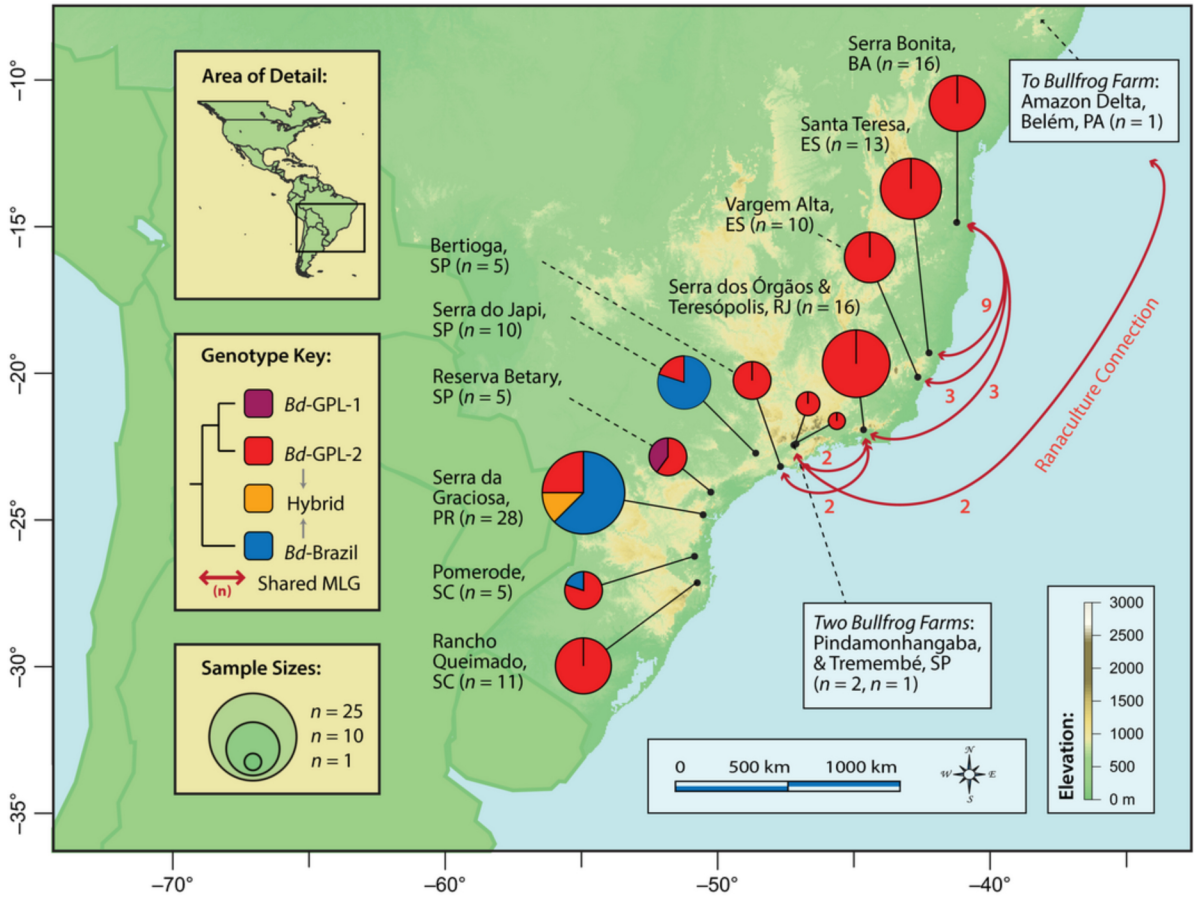

Figure 2-1: Spatial distribution of *Bd* genotypes in the Atlantic Forest. *Bd*-GPL-1, *Bd*-GPL-2, hybrid, and *Bd*-Brazil genotypes at collection sites along a 2400 km transect of the Atlantic Forest of Brazil. Diameters of pie graphs represent sample sizes. A hybrid zone is evident in Serra da Graciosa, Paraná. One site; Serra do Japi, São Paulo supports a higher frequency of Brazilian endemic genotypes than any other sample site in the Atlantic Forest. Red arrows indicate shared multilocus genotypes inferred from 12 markers and the total number of clonal isolates recovered.

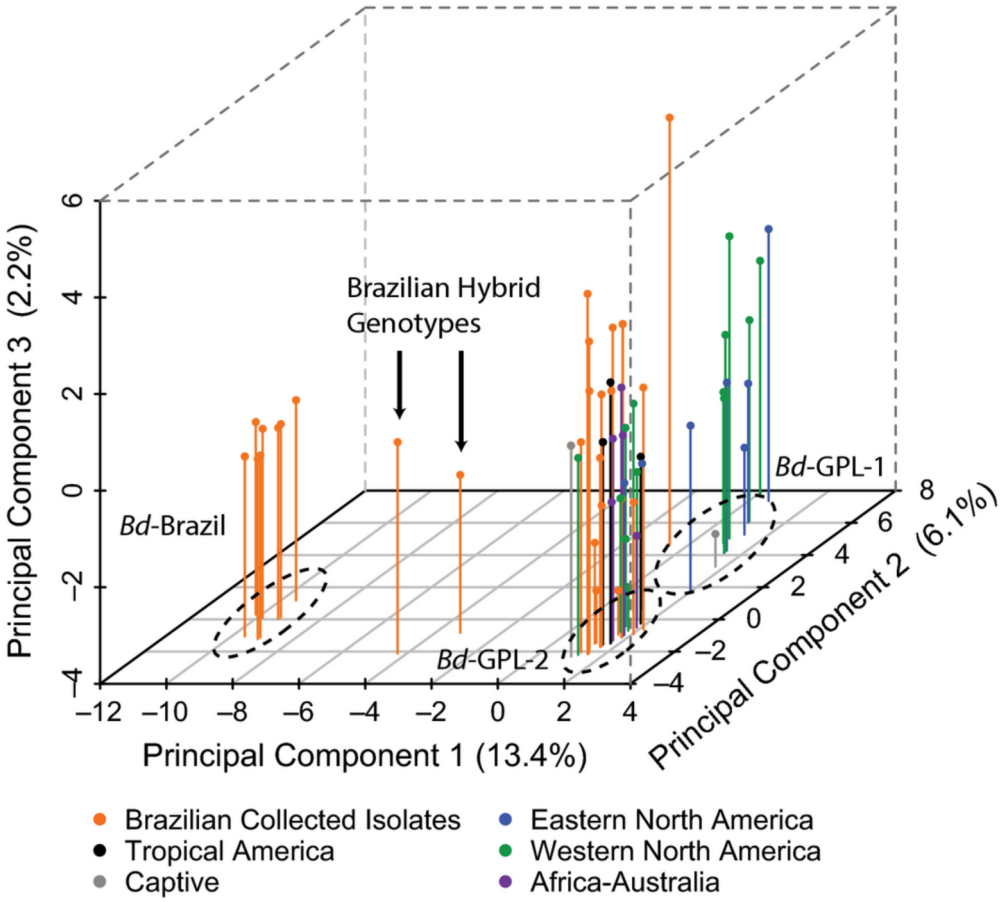

Figure 2-2: Principal components plot of global *Bd* representatives. Ordination plot of a global panel of *Bd* representatives from this and previously published studies, for which 10 multilocus sequence typing markers have been sequenced. Brazilian Atlantic Forest multilocus genotypes are highlighted in orange and show the greatest degree of genetic diversity of any sampled global region. The major lineages *Bd*-Brazil, *Bd*-GPL-1, and *Bd*-GPL-2 are outlined. The three major principal components explain 21.7% of currently sampled genetic variation in *Bd*.

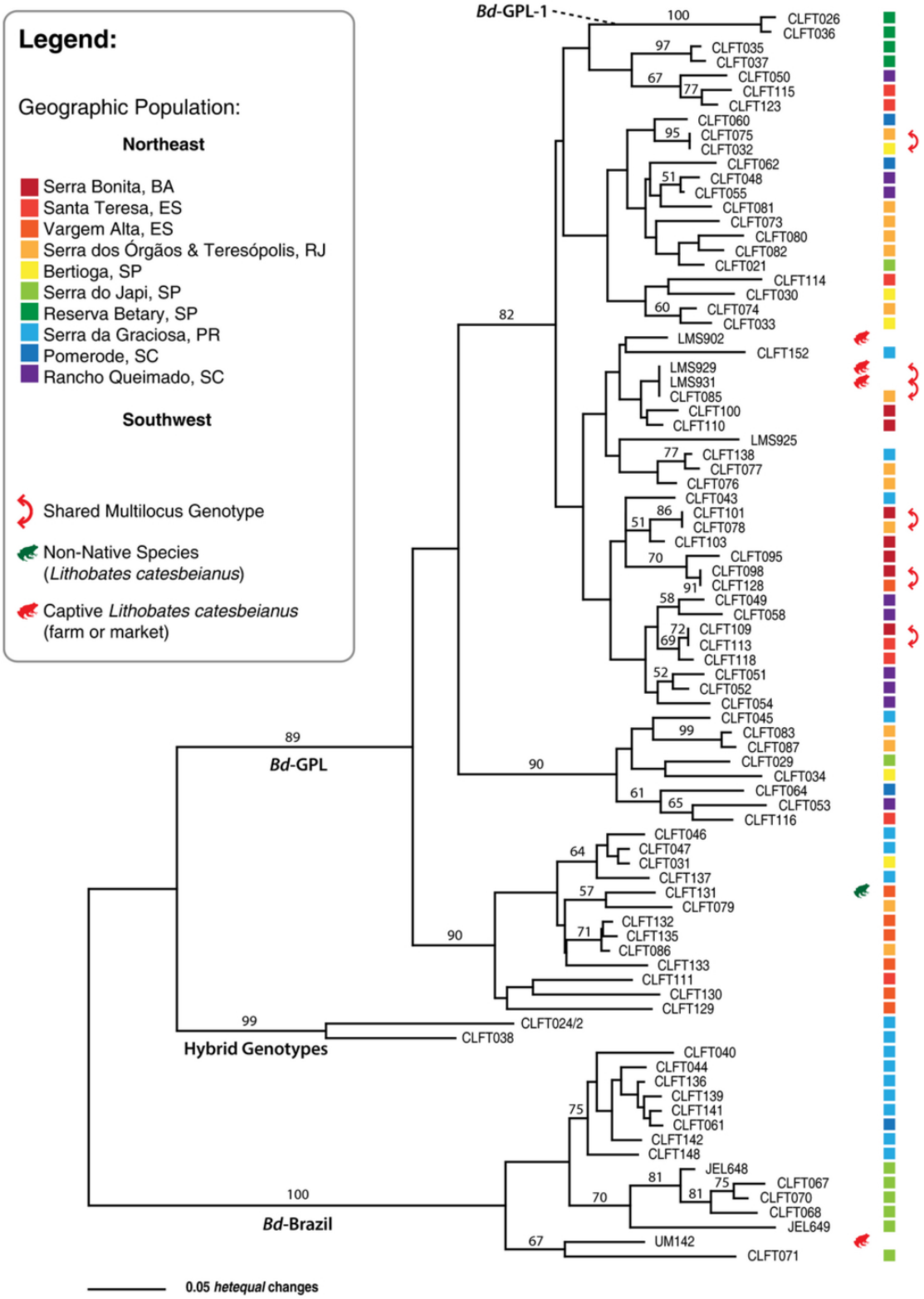
Legend:
Geographic Population:
Northeast
Serra Bonita, BA
Santa Teresa, ES
Vargem Alta, ES
Serra dos Órgãos & Teresópolis, RJ
Bertioga, SP
Serra do Japi, SP
Reserva Betary, SP
Serra da Graciosa, PR
Pomerode, SC
Rancho Queimado, SC
Southwest
Shared Multilocus Genotype
Non-Native Species (Lithobates catesbeianus)
Captive Lithobates catesbeianus (farm or market)
Bd-GPL-1
Bd-GPL
Hybrid Genotypes
Bd-Brazil
0.05 hetequal changes

Figure 2-3: Neighbor-joining dendrogram of Brazilian Atlantic Forest genotypes. Genotypes are based on 12 multilocus sequence typing markers, using a *hetequal* distance matrix. Genotypes are labeled with a representative isolate. Nodes leading to major lineages indicated (*Bd*-Brazil, *Bd*-GPL-1, and *Bd*-GPL-2). Collection localities are indicated by a color scale. Shared multilocus genotypes are indicated by curved arrows, and isolates from non-native and captive hosts are marked with green and red icons, respectively. Nodes with bootstrap support greater than 50% across 1000 bootstrap replicates are indicated.

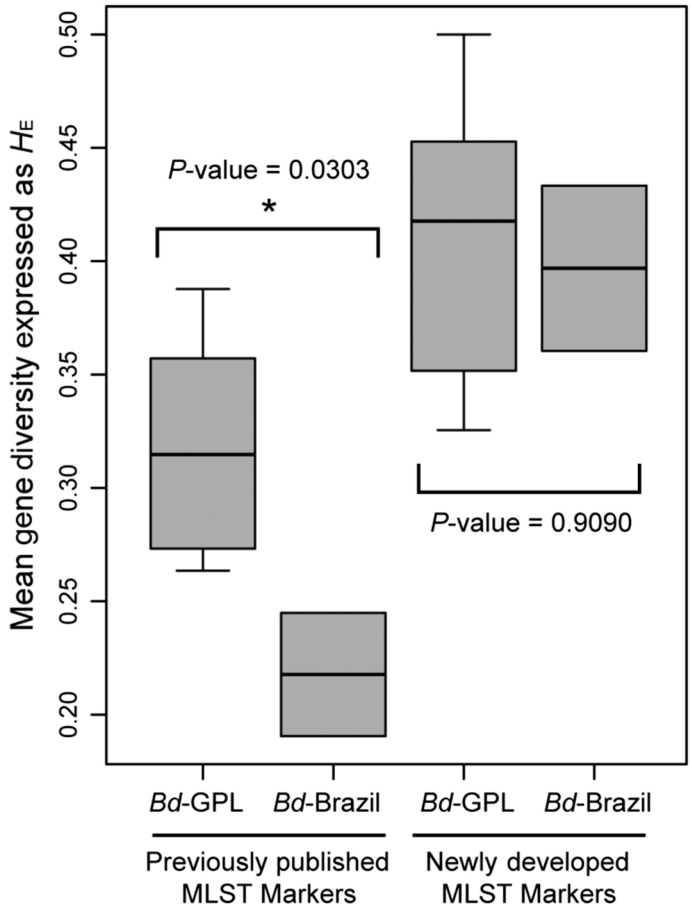

Figure 2-4: Comparison of previously published and newly developed markers. Previously published markers show bias toward capturing variation in the *Bd*-GPL lineage. Mean gene diversity differs significantly among lineages only when calculated separately using previously published markers (Wilcoxon rank-sum test), but significant differences are not observed when newly developed markers are analyzed separately.

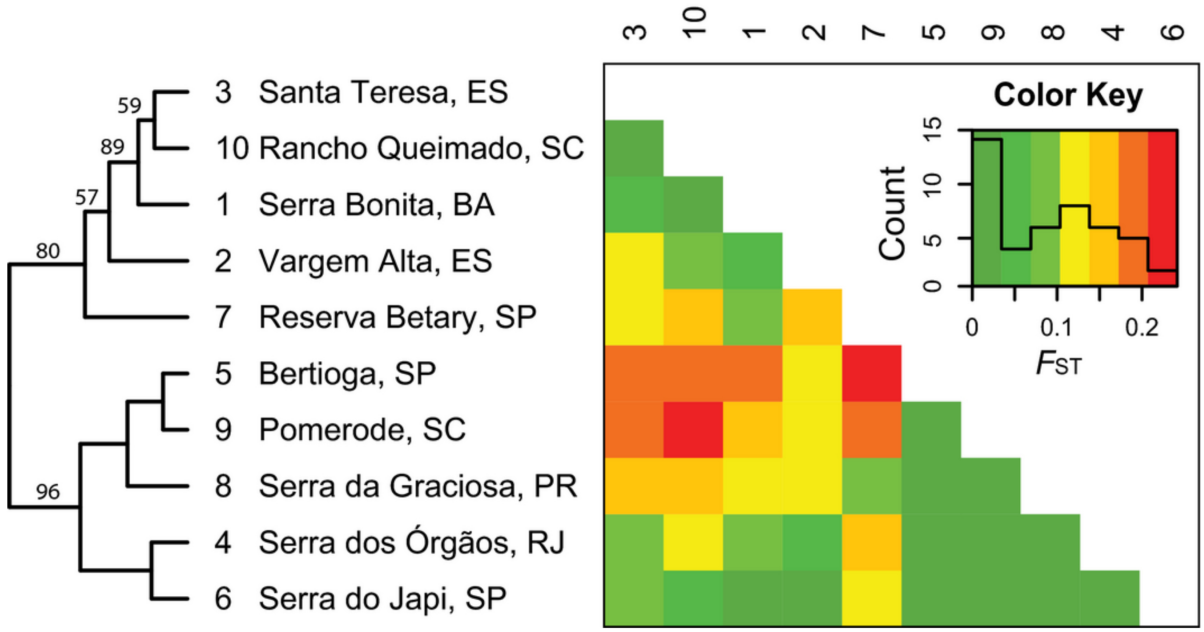

Figure 2-5: Heatmap of the pairwise F_{ST} matrix between *Bd*-GPL populations. The neighbor-joining dendrogram to the left shows inferred relationships between sample populations based on genetic differentiation. Population labels are numbered from northernmost (1) to southernmost (10) localities. Greener colors indicate low population differentiation (F_{ST} closer to zero), increasing to red to indicate greater population differentiation. Dendrogram nodes with bootstrap support greater than 50% across 1000 bootstrap replicates are indicated.

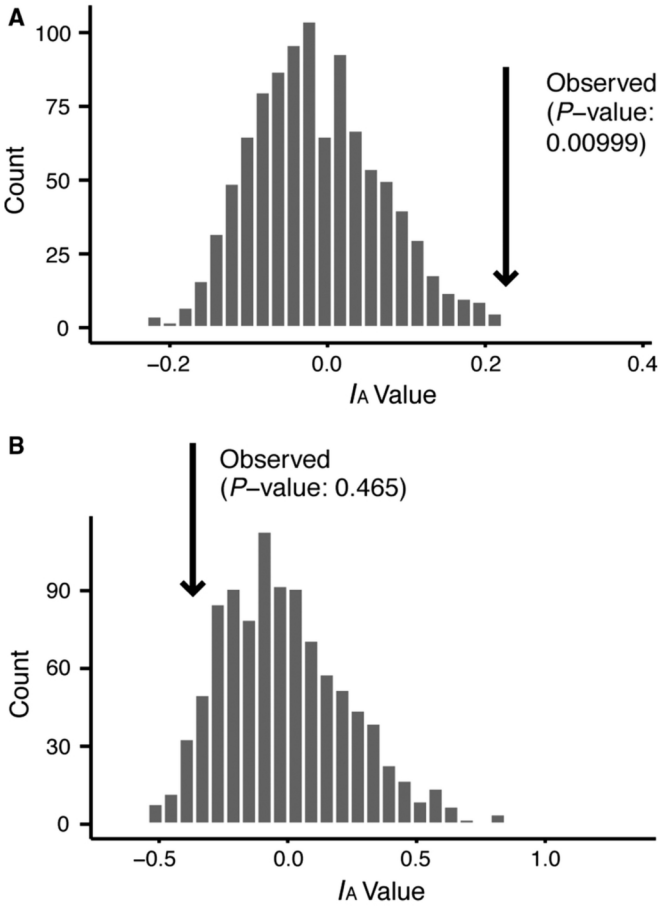

Figure 2-6: Histograms of simulated I_A values. Simulated index of association from 1000 permutations of randomization tests under a null model of allelic recombination, and observed values of I_A (indicated by arrows) for the *Bd*-GPL (A) and *Bd*-Brazil lineages (B). *P*-values correspond to the results from the random permutation test comparing observed indices to the distribution of simulation results.

Table 2-1: MLST marker details for loci analyzed for this study

Locus	Genomic Position	Alleles	PCR Primers	Anneal Temp.	Source
8009X2	**1:** 0.64 Mbp	4	F: 5'-TCGTGAAGAGCTTGGAAAGTCG-3' R: 5'-AGTTCTGTCGTCAATGCTGTAGGG-3'	54 °	Morgan *et al*. 2007
BdC5	**1:** 1.45 Mbp	3	F: 5'-TAATAGCGCCGACCGAACTA-3' R: 5'-ATGCCAAACCATGAGCAAAT-3'	54 °	James *et al*. 2009
BdSC2.0	**2:** 0.06 Mbp	4	F: 5'-TCAAGGTGCGTTTGCTAGTG-3' R: 5'-GCACTTACTGTTGGCAGCTTT-3'	60 °	New, this study
BdSC3.1	**3:** 0.17 Mbp	3	F: 5'-CAGTGACTTGCATCCACGAG-3' R: 5'-AATCGCTTCAACCAAACTGG-3'	54 °	Schloegel *et al*. 2012
BdSC4.16	**4:** 1.64 Mbp	3	F: 5'-TCAACTGGCTTTGAGCACAC-3' R: 5'-ATAGAGCATGCAGATCGCTTT-3'	54 °	Schloegel *et al*. 2012
R6046	**5:** 1.22 Mbp	2	F: 5'-CTATCTGCGCTCCCGTGTCAA-3' R: 5'-AGGGCTGCAACAACTGGATTT-3'	54 °	Morehouse *et al*. 2003
BdSC6.8	**6:** 0.87 Mbp	5	F: 5'-CCACTTCAAACACGTTCATTG-3' R: 5'-GTTTGGATTCCGAATGCTTG-3'	60 °	New, this study
BdSC6.15	**6:** 1.51 Mbp	4	F: 5'-GACGATAAAACGACAACAATCG-3' R: 5'-CCCTTTTTAGGTTGGCTTGC-3'	54 °	Schloegel *et al*. 2012
BdSC7.6	**7:** 0.66 Mbp	2	F: 5'-TGTGCCCGTGTTTTTGATTA-3' R: 5'-GTTACAACCTCCCGCTCGTA-3'	54 °	Schloegel *et al*. 2012
BdSC9.1	**9:** 0.19 Mbp	5	F: 5'-ACTGCTCACAGCACTTCGAC-3' R: 5'-CAAACCGTTGTACGAACCAG-3'	60 °	New, this study
BdSC11.5	**11:** 0.53 Mbp	2	F: 5'-CGGATCCGTTCTTCATTTTG-3' R: 5'-AAAGAGAATTCAACGAGAGCAA-3'	60 °	New, this study
BdSC16.2	**16:** 0.27 Mbp	2	F: 5'-CCGTACGATAGAGCGTTAGCA-3' R: 5'-TGCCCATATTCATGGACTGA-3'	60 °	New, this study

Genomic positions are based on Broad Institute reference genome assembly of JEL423 (version 17-Jan-2007).

Table 2-2: Atlantic Forest *Bd* populations sampled for this study, respective sample sizes (n), and indices of genetic diversity

					Clone-Corrected Data	
Populations:	**n**	**MLGs**	**Mean Allele Richness**	**Genotypic Diversity**	**Observed Heterozygosity (H_O)**	**Expected Heterozygosity (Gene Diversity, H_E)**
1. Serra Bonita, BA	16	7	1.667	0.438	0.536	0.330
2. Santa Teresa, ES	13	7	1.667	0.539	0.429	0.306
3. Vargem Alta, ES	10	7	1.750	0.700	0.500	0.350
4. Serra dos Órgãos & Teresópolis, RJ	16	14	1.750	0.875	0.512	0.369
5. Serra do Japi, SP	10					
Bd-GPL	2	2	1.667	1.000	0.500	0.417
Bd-Brazil	8	6	1.583	0.750	0.403	0.292
6. Bertioga, SP	5	5	1.667	1.000	0.417	0.328
7. Reserva Betary, SP	5	4	1.833	0.800	0.458	0.417
8. Serra da Graciosa, PR	26					
Bd-GPL	7	7	1.917	1.000	0.488	0.406
Bd-Brazil	16	7	1.750	0.438	0.440	0.293
Hybrids	3	2	2.083	0.667	0.750	0.569
9. Pomerode, SC	5					
Bd-GPL	4	3	1.667	0.750	0.417	0.350
Bd-Brazil	1	1	1.417	1.000	0.417	0.417
10. Rancho Queimado, SC	11	9	1.667	0.818	0.444	0.304
All *Bd*-GPL	**89**	**61**		**0.685**	**0.475**	**0.374**
All *Bd*-Brazil	**25**	**14**		**0.583**	**0.423**	**0.287**
Global	**117**	**77**		**0.658**	**0.473**	**0.511**

Populations of enzootic and hybrid lineages are shaded gray.

Table 2-3: Population genetic indices, and results of Hardy-Weinberg exact tests. Inbreeding coefficients and indices of association after clone-correction, with associated *P*-values from the results of Hardy-Weinberg exact tests and random permutation tests under a model of random recombination

	Hardy-Weinberg Exact Test		Index of Association Permutation Test	
Population:	F_{IS}	***P*-Value**	I_A	***P*-Value**
1. Serra Bonita, BA	-0.709	**< 0.0001**	-0.291	0.8111
2. Santa Teresa, ES	-0.450	**0.0016**	0.346	0.1728
3. Vargem Alta, ES	-0.482	**0.0003**	0.086	0.3287
4. Serra dos Órgãos & Teresópolis, RJ	-0.406	**< 0.0001**	0.281	0.0629
5. Serra do Japi, SP				
Bd-GPL	-0.333	0.3500	*Permutation test not conducted* (n < 3)	
Bd-Brazil	-0.436	**0.0068**	0.179	0.2577
6. Bertioga, SP	-0.316	0.0547	-0.019	0.4595
7. Reserva Betary, SP	-0.119	0.2857	3.38	**0.0020**
8. Serra da Graciosa, PR				
Bd-GPL	-0.224	0.1534	1.063	**0.0050**
Bd-Brazil	-0.569	**0.0001**	-0.009	0.4386
Hybrids	-0.565	**0.0382**	*Permutation test not conducted* (n < 3)	
9. Pomerode, SC				
Bd-GPL	-0.250	0.2726	-0.556	0.6623
Bd-Brazil	*Exact test not conducted* (n=1)		*Permutation test not conducted* (n < 3)	
10. Rancho Queimado, SC	-0.506	**< 0.0001**	0.517	**0.0290**
All *Bd*-GPL	-0.245	**< 0.0001**	0.226	**0.0099**
All *Bd*-Brazil	-0.416	**0.0012**	-0.013	0.465
All Populations	0.074	**< 0.0001**		

P-values in bold indicate significant deviations from null expectations under Hardy-Weinberg equilibrium and a model of random recombination, respectively. Populations of enzootic and hybrid lineages are shaded gray.

Chapter 3

Population genomic sequencing reveals deep divergence and hybridization between *Batrachochytrium dendrobatidis* lineages

Abstract:

Emerging wildlife diseases caused by fungi are on the rise in recent decades. Chytridiomycosis is one example that has been implicated in recent population declines and extinctions of frog species worldwide. The disease is caused by *Batrachochytrium dendrobatidis* (*Bd*). Here we investigate the genomic patterns of lineage divergence and hybridization between two distinct lineages of *Bd*. The recent discovery of hybrid zone is significant because *Bd* was once thought to be a strictly asexually reproducing pathogen. The only known cases of hybridization in this species are restricted to this narrow zone on the Atlantic coast of Brazil. Natural hybrids present a valuable opportunity to understand the genetic basis of lineage divergence, and the first steps toward speciation. To understand the accrued genetic incompatibility between the divergent parental lineages, we obtained Illumina whole-genome sequences from 51 Brazilian Atlantic Forest strains of *Bd* – including four hybrids and broad geographic representation from both the *Bd*-GPL and *Bd*-Brazil parent populations. We assessed hybrid genomes for genetic incompatibilities by scanning hybrids for unequally inherited alleles from known parental alleles. Loci with unequally inherited alleles occurred in clustered blocks throughout the hybrid genomes and shared overlap with divergence islands we identified by outlier F_{ST} analysis. Gene function

analyses showed that these divergent loci were enriched for genes involved in response to oxidative stress, suggesting a potential mechanism underlying the ecological diversification between *Bd* lineages.

Introduction:

Incidences of novel wildlife diseases are on the rise around world (Daszak *et al* 2001; Jones *et al* 2008). In many cases, the factors contributing to their emergence are unknown. Predicting the dynamics of infectious wildlife diseases is critical to both environmental sustainability and human health. Of these novel wildlife diseases, an increasing proportion is caused by fungal pathogens in particular (Fisher *et al* 2012). As eukaryotes the basic biology, and population dynamics of fungal pathogens are distinct from those of better-known bacterial and viral pathogens. However, few studies exist that address the evolutionary ecology of emerging fungal diseases, and the role of population history – such as geographic invasion – in driving the emergence of novel diseases.

Chytridiomycosis is the emerging infectious disease implicated in recent, conspicuous amphibian declines and extinctions worldwide (Berger *et al* 1998, Rachowicz *et al* 2006). This disease is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*, Longcore *et al* 1999). Appearing enigmatically in the late 20th century, *Bd* quickly became one of the most significant threats to global amphibian biodiversity (Skerratt *et al* 2007, Fisher *et al* 2009). In many regions of the globe *Bd* is newly introduced and actively spreading (*e.g.* Lips *et al* 2006, Vredenburg *et al* 2010, Walker *et al* 2010, Cheng *et al* 2011). Genotype analyses of *Bd* populations from these regions have revealed that introduced pathogen strains all belong to a

single, rapidly expanding clonal lineage (James *et al* 2009), termed *Bd*-GPL (for *Global Pandemic Lineage*, Farrer *et al* 2011).

We recently described the spatial structure of another deeply divergent *Bd* lineage in a field study of wild pathogen strains (Jenkinson *et al* 2016). Our study showed a restricted distribution of the putative Brazilian endemic *Bd*-Brazil strain with a region of spatial overlap between *Bd*-GPL in the southwestern region of the Atlantic Forest (Figure 2-1). We also collected hybrid strains that were the result of outcrossing events between *Bd*-GPL and *Bd*-Brazil in the Serra da Graciosa mountain range, Paraná State. The genetic patterns in our earlier study showed that *Bd*-GPL had either recently expanded in range, or recently shared migrants between geographic populations. The spatial distribution of these lineages in the Atlantic Forest suggested that the endemic *Bd*-Brazil was in the process of being competitively excluded from its former range.

The *Bd*-GPL invasion hypothesis we outlined leads to immediate next questions about the history, and the future consequences of this contact. If the globally expanding *Bd*-GPL lineage is also invasive to southern Brazil – was it transported to the region by human activity? And if so, what are the ecological and evolutionary consequences of this secondary contact between strains of a destructive pathogen? Two possible outcomes of secondary strain contact are: competition (if habitat needs sufficiently overlap), or mixing and coexistence through sexual outcrossing (if the strains are not exceedingly diverged). We have already observed outcrossing between lineages. With multilocus sequence data however we were not entirely conclusive whether the few hybrid strains that had been observed in Brazil were of independent origin. Hybrids are rare

in this region, rarer than expected based on parental population frequencies in the contact zone (Figure B-1). If ongoing hybridization is occurring in this zone, we suggested that the few surviving strains in the wild represent successful allele combinations out of a potential many others that were not viable because of genetic incompatibility. Consequently, these hybrid strains represent a unique opportunity to probe the nature of lineage divergence by differential adaptive selection. As lineages diverge, incompatibilities accumulate through drift and differential selection (Dobzhansky 1940, Coyne and Orr 2004). We predict that due to a historical lack of contact, these incompatible loci can inform the nature of divergence in *Bd*. If this is true, parental alleles disproportionately inherited in hybrid offspring may provide clues to the environmental selective pressures favoring one parent or another.

The other potential outcome of secondary strain contact is conflict over resources and competition between lineages (discussed further in Chapter 4). Each of these potential outcomes carries consequences for virulence evolution in *Bd*. An understanding of population history is crucial to understanding the range of potential consequences. For example, strain histories can inform the differential selective environments to which lineages are locally adapted. The inference of strain history can also inform the timing, source, and patterns of invasive spread, all of which contribute to understanding the ecological and evolutionary trajectory of invasive pathogens. Unanswered questions about the population history of these co-occurring lineages include: what was the timing and geographic origin of an invasion? What regions of the genome underlie divergence and differential adaptation in the incipient stages of isolation. And also, what happens when that external forces disrupt incipient isolation in allopatry, in this case via human pathogen translocation.

Given these open questions, we revisited 44 of our previously collected field isolates from nine geographic populations in the Atlantic Forest for whole genome resequencing. Population genetic studies of *Bd* have traditionally been hindered by low genetic variation between strains (Morehouse *et al* 2003), and the tendency for variation to be obscured by loss of heterozygosity (LOH) (James *et al* 2009). Recent studies have shown Illumina genome resequencing to be an effective tool for overcoming these challenges in *Bd* (Farrer *et al* 2013). We also returned to the Serra da Graciosa hybrid locality (Schloegel *et al* 2012; Jenkinson *et al* 2016) to further sample isolates from a wider area up to 25 km from the originally collected hybrid strains. After screening new isolates by multilocus sequencing (described in Chapter 2), we selected five of these new hybrid zone strains for genome sequencing. Finally, we opportunistically collected two more isolates from Campos do Jordão, Minas Gerais that we sequenced because of their collection proximity to known clusters of bullfrog farming in Brazil (Schloegel *et al* 2012).

With this dataset, our study aimed to address the overarching hypothesis that *Bd*-GPL is recently introduced to the Atlantic Forest, and is expanding in range by competitively excluding *Bd*-Brazil. Specifically we addressed this hypothesis by: 1) determining the fine scale relationships and variation among our Atlantic Forest isolates, 2) determining regions of genomic divergence, 3) assessing divergence times for the Atlantic Forest lineages, and 4) interrogating hybrid genomes for unequally inherited alleles. With this study we aim to elucidate the genetic nature of lineage divergence in *Bd* and reconstruct the events culminating in the pattern of lineage co-occurrence we see in today's Atlantic Forest habitat.

Materials and Methods:

We sampled 51 *Bd* isolates from the Brazilian Atlantic Forest for whole genome resequencing (Table 3-1). Of the sampled isolates, 44 were previously collected (Jenkinson *et al* 2016), 5 were newly sampled from the area surrounding the Serra da Graciosa hybrid zone in the state of Paraná (Figure B-1), and 2 were sampled from Campos do Jordão, in the Serra da Mantiqueira Mountains, Minas Gerais. The field collection methods are described in Jenkinson *et al* (2016). *Bd* isolates were maintained on 1% tryptone agar plates at 20-21º C until sufficient growth had occurred for DNA extraction. We isolated genomic DNA as soon as possible after isolation in order to minimize laboratory passages in culture (Refsnider *et al* 2015). DNA was extracted from all samples after two to three passages. We extracted DNA from isolates using a modified CTAB miniprep with chloroform and isoamyl alcohol (Zolan & Pukkila 1986). We quantified DNA concentration in our samples using the Qubit 2.0 Fluorometer with the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Inc). We used the results from this initial quantification to dilute all samples to the appropriate input concentration for library preparation with the Nextera XT DNA Library Prep Kit (Illumina Inc).

We prepared short-insert (~450bp) DNA fragment libraries according to the Nextera XT manufacturer's recommendations with slight modifications. Briefly, we input 1ng of quantified template DNA (diluted to 0.2ng/μL) from each sample. The Nextera method utilizes an active transposome to simultaneously fragment and adapter tag (tagment) double-stranded template DNA. We carried out the tagmentation step at 55º C for 5 minutes before neutralizing the transposome. Then we carried out a limited-cycle PCR to amplify and index the tagmented

DNA. We dual indexed individual samples for paired-end sequencing using the Nextera XT v2 Index Kit. Post-PCR, we purified libraries by ligating indexed fragments to AMPure XP magnetic beads (Beckam Coulter Inc), and washing away impurities while retaining the beads with an Agencourt 96-well ring magnet plate (Beckam Coulter Inc). We quality checked the fragment libraries for appropriate size and concentration with an Agilent 2100 Bioanalyzer (Agilent Technologies Inc). After quality control, we normalized and pooled the libraries for sequencing the Illumina HiSeq 2500 platform (Illumina Inc). Our samples were paired-end sequenced for 125 cycles across two HiSeq 2500 flow cell lanes by the University of Michigan Core DNA Sequencing Laboratory.

We demultiplexed the resulting Illumina reads and assessed read quality metrics for each sample using FASTQC (Andrews 2010). We trimmed sequencing adapters and low quality bases from the reads as needed with TRIMMOMATIC (Bolger *et al* 2014). We assembled our reads to the *Bd* reference genome generated from strain JEL 423 (Broad Institute, ver. Jan. 2007) with BWA-MEM (Li 2013). After assembly to reference, we sorted and removed duplicate reads with PICARD (Broad Institute). We realigned indels, recalibrated read quality scores using the dataset from Rosenblum *et al* (2013), and indexed reads with the Genome Analysis Toolkit suite of tools (GATK; McKenna *et al* 2010). We identified SNP and indel variants with GATK HAPLOTYPECALLER and performed the final joint genotyping with GATK GENOTYPEGVCFS. Finally, we selected and quality filtered SNPs (quality > 50) to produce a final, high-confidence panel of 90,429 SNPs with GATK VARIANTFILTRATION.

We determined average and per-SNP sequencing coverage for our samples with GATK COVERAGEWALKER. We used custom perl scripts to determine genomic heterozygosity (H_O), and local average heterozygosity across a 50kb sliding window advancing every 10kb for each isolate. We also used custom scripts to determine genetic distances among our panel of isolates under a *hetequal* character transition matrix – assuming heterozygous polymorphisms at each SNP to be one step from the nearest heterozygote and two steps from other heterozygotes (Mountain & Cavalli-Sforza 1997). We visualized the calculated distances as a neighbor-joining dendrogram using PHYLIP (Felsenstein 2005). We evaluated statistical support for our *Bd* tree by resampling 100 bootstrap pseudo-replicates from our SNP data for distance analysis with a custom perl script. We looked for cross-lineage hybrids by conducting a population structure analysis in FASTSTRUCTURE (Raj *et al* 2014) with K = 2 corresponding to the parental *Bd*-GPL and *Bd*-Brazil lineages. We calculated F_{ST} (Weir & Cockerham 1984) between *Bd*-GPL and *Bd*-Brazil populations in non-overlapping 10kb sliding windows with VCFTOOLS (Danecek *et al* 2011). We conducted an analysis of gene ontology (GO) enrichment in outlier loci with the GOstats package (Falcon & Gentleman 2007) using the Broad Institute functional annotation of the *Bd* reference genome (JEL423).

To estimate relative divergence times of the *Bd*-GPL and *Bd*-Brazil Atlantic Forest populations, we generated variant calls separately for the *Bd* mitochondrial genome. We used the mitochondrial genomes to infer divergence times because mitochondria are not subject to recombination (both sexual and mitotic) as nuclear genomes are, and because mitochondria are uniparentally inherited in *Bd* (discussed below). Our short reads were assembled to the reference mitochondrial genome and processed as above to generate a final variant call file as above. We

exported SNP calls (550 SNPs) and invariant sites from the mitochondrial reference to fasta format and constructed a nexus dataset for our mitochondrial genomes. We used BEAST (Drummond & Rambaut 2007) to sample from the posterior density of time calibrated trees for our 51 Atlantic Forest strains. We applied a constant-size coalescent tree prior and a strict molecular clock with a rate of 0.9×10^{-9} substitutions per site per year, a general substitution rate previously estimated for fungi (Kasuga *et al* 2002). We allowed the Markov chain Monte Carlo (MCMC) sampling to burn in for the first 10 million generations, then we sampled every 1,000 generations for 20 million generations.

Viable hybrids between divergent strains can serve as a valuable resource to examine divergence and hybrid incompatibility between parental lineages (Orr 1995, Johnson 2010). We investigated the genomes of hybrid strains for unequally inherited alleles suggestive of loci with Dobzhansky-Muller incompatibilities. We performed this analysis with a custom perl script. First the script identified loci that were reciprocally fixed in each parental population (*Bd*-GPL and *Bd*-Brazil). Then the script assessed each hybrid genome and identified homozygous positions at these loci, and recorded the parent population inferred as the allelic donor. All in-house scripts used for data analysis in this study are available online from (https://github.com/Michigan-Mycology).

Finally, to assess the placement of our Brazilian samples within the global context we downloaded a global panel of 49 previously published genomes representing close to all known *Bd* isolates that have been whole genome sequenced (NCBI Short Read Archive Project IDs: SRP017502 and SRP017570; Farrer *et al* 2013; Rosenblum *et al* 2013; Table B-1). These

downloaded reads were aligned to the JEL423 nuclear reference genome, then processed and variant called as above. We ran our distance script on the resulting SNP matrix (83,404 SNPs), then constructed a neighbor-joining dendrogram with PHYLIP and assessed branch support with 100 bootstrap replicates as above.

Results:

After processing, assembly, and quality filtering, we achieved a mean sequencing depth of 26.45x per isolate (range = 15.72x - 53.12x coverage; Table 3-1). The final variant call dataset for our 51 newly sequenced isolates consisted of 90,429 SNPs, corresponding to approximately 0.38% variable sites across the 24Mb *Bd* genome.

Atlantic Forest isolate relationships

The genealogical relationship between the Brazilian Atlantic Forest isolates showed deep divergence between two well supported *Bd*-GPL and *Bd*-Brazil lineages (Figure 3-1). Our neighbor-joining analysis also show the placement of hybrid genomes, and the genetic distance between them. One of these hybrid genomes, CLFT039, is a hybrid isolate previously described from our Brazil transect survey (Jenkinson *et al* 2016). The two others are newly discovered hybrid isolates collected from within 25km of the originally described *Bd*-GPL x *Bd*-Brazil hybrid isolate of Schloegel *et al* (2012). Our FASTSTRUCTURE analysis showed that the inferred admixture proportions of the hybrid strains CLFT039 and CLFT160 are consistent with F1 hybrids, and the admixture pattern of isolate CLFT165 is consistent with an F2 backcross to the *Bd*-Brazil parent population.

Both major lineages were supported with 100% bootstrap support (BS). We observe varying degrees of geographic structure within the two major lineages. In the *Bd*-Brazil clade, our isolates largely cluster by geography with the exception of isolate CLFT 144. In the *Bd*-GPL clade, we observe our two representative members of the *Bd*-GPL1 subclade (Schloegel *et al* 2012) cluster together with 100% BS. The rest of our Atlantic Forest isolates group largely by geography with a number of notable exceptions. For example, we observe isolate CLFT157 from the Serra da Graciosa hybrid zone resolved in a well-supported clade with all the sampled isolates from Serra Bonita, Bahia, a geographic population separated by 1500km.

Patterns of heterozygosity

The average genomic heterozygosity of *Bd*-GPL isolates was significantly greater than the average heterozygosity of *Bd*-Brazil (mean proportion heterozygous SNPs, *Bd*-GPL: 0.307, *Bd*-Brazil: 0.163; Welch's $t = 15.48$, $p < 0.001$; Figure 3-2). Our backcrossed F2 hybrid showed the lowest proportion of heterozygous SNPs among the hybrids (0.397) and the mean proportion of heterozygosity in the two F1 hybrids was (0.644). All isolates showed long runs of homozygosity. Consistent with the hypothesis that these runs of homozygosity are the result of mitotic crossing over, or break-induced repair, all major runs of homozygosity extend to the nearest end of the chromosome on which they occur (Figure B-2). Visual inspection of individual heterozygosity plots showed that *Bd*-Brazil individuals shared only a single long run of LOH among individuals (on the left arm of chromosome 1 approximately 1Mbp long) despite showing lower overall heterozygosity than *Bd*-GPL. In contrast, the *Bd*-GPL strains showed many more pronounced runs of LOH, and many of which are shares across several individuals. Two major LOH events were shared by all of our *Bd*-GPL isolates, one approximately 2Mb long

on the right arm of chromosome 1, and another run approximately 2Mb on the right arm of chromosome 2. The other major LOH event observed was a run approximately 1Mb on the left arm of chromosome 1 shared by 11 isolates. Those isolates with major LOH runs on both the left and right arms of chromosome 1 were almost completely homozygous across the entire chromosome (*e.g.* isolate CLFT120; Figure B-2).

Divergent genomic regions

The average F_{ST} between *Bd*-GPL and *Bd*-Brazil populations in 10kb sliding windows was high (mean = 0.677, S.D. = 0.211) underscoring the deep divergence between these lineages. From the 2362 windows examined, we selected the top 5% of the most divergent windows (with the highest cross lineage F_{ST} values) for further examination (Figure 3-3). These 5% of windows were largely clustered in large blocks of divergence across the *Bd* genome. The largest of these divergence blocks fell within the right arm of chromosome 1, across chromosome 2, on the left arm of chromosome 4, and on the right arm of chromosome 8. The divergence blocks on chromosomes 1 and 2 correspond to the major LOH runs described above. We extracted the genomic coordinates of these top 5% windows of F_{ST} divergence and performed a GO enrichment analysis for overrepresented GO functional groups. These divergence blocks in the *Bd* genome were enriched for molecular functions related to – among others – superoxide dismutase activity ($p < 6E-4$), and multiple genes encoding proteins involved in oxidoreductase activity ($p < 6E-4$, and $p = 5.2E-3$). The major functional groups enriched in our inferred divergence regions are shown in Figure 3-4 (full results of the GO analysis are presented in Table B-2).

Divergence time of the Atlantic Forest populations

We constructed mitochondrial genome alignments from our filtered SNP variants. The aligned mitochondrial dataset was ~170kb in length with 550 variable positions. Using a universal substitution rate we inferred that the tree height for the entire *Bd* tree of Atlantic Forest isolates is approximately 94.13 KYA (95% C.I. 83.18 - 105.33 KYA; Figure 3-5). We inferred the time to most recent common ancestor of the *Bd*-GPL isolates in Brazil to be approximately 2.06 KYA (95% C.I. 1.35 - 2.92 KYA). Finally, we inferred the time to most recent common ancestor of the *Bd*-Brazil clade to be approximately 2.57 KYA (95% C.I. 1.48 - 3.72 KYA).

Patterns of inheritance in the hybrid isolates

We found that 20,106 SNPs were reciprocally fixed between the *Bd*-GPL and *Bd*-Brazil parental lineages. This represents 22.2% of SNPs and approximately 0.08% of the 24Mb genome with fixed differences between the two lineages. The patterns of allele inheritance at these reciprocally fixed loci were unequal across all the hybrid isolates we examined. Unequal inheritance of the reciprocally fixed SNPs favored the *Bd*-GPL parental allele in both F1 hybrids (Figure 3-6). The average ratio of homozygous *Bd*-GPL to *Bd*-Brazil alleles at these fixed SNPS was 1.85 to 1. The F1 isolate CLFT039 had 3566 homozygous SNPs at these sites at a ratio of 2.36 : 1 (*Bd*-GPL to *Bd*-Brazil) while F1 isolate CLFT 160 had 4176 homozygous SNPs at a ratio of 1.34 : 1. Patterns of unequal inheritance were markedly different in CLFT165 – the F2 backcross to *Bd*-Brazil. The F2 isolate was homozygous for 18,038 of the reciprocally fixed SNPs, corresponding to approximately 89.7% of the fixed sites. In contrast to the pattern observed in our F1s, unequal inheritance disproportionately favored *Bd*-Brazil alleles at a ratio of 1 : 30.70 (*Bd*-GPL to *Bd*-Brazil). To discern the pattern of mitochondrial inheritance in sexual

hybrids we constructed a neighbor joining tree of mitochondrial genomes as above. This analysis revealed that mitochondria in *Bd* are uniparentally inherited. This mitochondrial tree showed that both F1 hybrids inherited mitochondrial genomes from their *Bd*-GPL parents, and our F2 hybrid inherited its mitochondrial genome from the *Bd*-Brazil parent in the backcross.

Meta-analysis of published strains

We compiled our isolate sequences with a panel of previously published *Bd* genomes to test the hypothesis of *Bd*-GPL introduction to the Atlantic Forest, and to infer global geographic affinity of our isolates, if any exists. The genealogical relationships among this global panel of 100 *Bd* isolates showed of 49 of our 51 Atlantic Forest isolates falling in a near-polytomous clade of global isolates with 90% BS support. Our Atlantic Forest isolates did not group together in significantly supported clades, nor did they clade significantly with *Bd*-GPL strains (in subclade *Bd*-GPL2) isolated elsewhere. Other globally isolated strains in this 90% supported clade were collected from Australia (Southeast), Bolivia, Colombia, Italy (4 isolates), Japan, Panama (3 isolates), Puerto Rico, South Africa (2 isolates), Switzerland (2 isolates), UK, and USA (West, 2 isolates). Our two known members of the GPL subclade *Bd*-GPL1 (identified by multilocus sequence typing described in Chapter 2) grouped with a previously identified Atlantic Forest representative of *Bd*-GPL1 (CLFT026 with 100% BS, Schloegel *et al* 2012), suggesting a single, separate introduction of *Bd*-GPL1 into Brazil. This group did not form a supported clade with any other *Bd*-GPL1 members in the analysis, however, not allowing the clear inference of a source population for this introduction.

Discussion:

Strain hybridization in the Brazilian Atlantic Forest

Here we present further insight into the strong genetic divergence between the *Bd*-GPL and *Bd*-Brazil lineages. Based on our analyses these lineages shared a most recent common ancestor on the order of 100,000 years before present. Finally, we provide further evidence that an active hybrid zone has established in a region of the Brazilian Atlantic Forest where the two lineages contact. These results significantly advance current working knowledge concerning the basic biology of an emerging pathogen recently thought to strictly reproduce asexually. We provide evidence based on the phylogenetic placement (Figure 3-1), and inheritance patterns of the F1 hybrids (Figure 3-6) that the hybrid strains are likely of independent origin. And for the first time, we show that cross-lineage hybrids of *Bd* are capable of backcrossing with parental populations in the wild. Ecologically based, post-mating fitness of hybrids will vary with habitat and potentially time (Schluter 2001), but in other natural populations (*e.g.* three-spine sticklebacks), backcrossed hybrids may be more fit than F1s if they occur in environmental conditions favorable to the backcrossed parent (Hatfield & Schluter 1999). This observation is also of potential concern to biodiversity conservation efforts because we know that it is possible for novel fungal pathogen species to arise from lineage hybridization (Stukenbrock *et al* 2012). We identify loci that may be driving hybrid incompatibility and incipient speciation in *Bd*. These loci should have been locally adapted to the environmental context of each parental lineage, and contribute to understanding the genetic factors underlying lineage divergence.

Patterns of genomic heterozygosity

In contrast to the rare sexual outcrossing we report here, the vast majority of *Bd* reproduction is asexual. Diploid pathogens tend to lose heterozygosity over long periods of

asexuality – and sexual outcrossing is the only way to rapidly regenerate heterozygosity. LOH most often occurs in clonal lineages through mitotic recombination, gene conversion, or other forms of chromosomal breakage and repair (James et al 2009). These processes often result in long runs of homozygosity to the end of a chromosome arm. If these regions contain alleles that are beneficial enough to the environmental pressures where they occur – especially if they are recessive or act in a dosage-dependent manner – selective sweeps can take the form of long (sometimes multiple Mb) genomic regions (Figure B-2: GPL strains, chromosome 2).

It was striking that heterozygosity plots showed more LOH regions in *Bd*-GPL than in *Bd*-Brazil, yet genome-wide average heterozygosity was significantly higher in *Bd*-GPL strains (Figure 3-2). The pattern of lowered *Bd*-Brazil heterozygosity countered our expectation that an endemic lineage should show higher heterozygosity than recently bottlenecked invasive lineages (Balloux *et al* 2003; De Meeus *et al* 2006). The important part of this signal may lie in the contrast between the high LOH observed among *Bd*-GPL isolates, and not observed in *Bd*-Brazil. Based on the shared terminal coordinates of these LOH regions we can infer their homology, and speculate that they are shared signatures of recent bottleneck events indicative of recent expansion. Other demographic factors such as rapid population growth of a successful invasive lineage, multiple introductions, and undersampling rare endemics because of recent extinctions could all contribute to the patterns we observe.

Geographic signal across genomes

Our results showed that *Bd*-GPL populations in Brazil were more geographically structured than previously thought. Although we still observe some clustering of isolates from

geographically distant populations (Bahia with Minas Gerais, and Bahia with Paraná; Figure 3-1) indicating recent gene flow or rapid expansion (Excoffier *et al* 2009). We document new occurrences of the north-temperate restricted GPL subclade *Bd*-GPL1 from the southern Paraná State, providing evidence of at least two potential introductions of *Bd*-GPL into the Atlantic Forest. Our meta analysis of published genomes was not able to resolve the deeper question of a source population. Clearly, a deeper global sampling effort is still required to reveal clear source populations for putative strain invasions. Our compilation of published genomes shows that many global regions remain undersampled particularly in the Asian continent, where a new novel genotype was recently described (*Bd*-Korea; Bataille *et al* 2013), and where the newly discovered sister species to *Bd* (*Batrachochytrium salamadrivorans*, Martel *et al* 2013) is hypothesized to be endemic. We anticipate that as global sampling of *Bd* genomes continues to grow, our genome sequences will contribute to wider comparative genomic studies like the one we attempted. A coordinated, collaborative network of research groups sampling strategically and sharing data may be the only way to shed light on the continually pressing mystery surrounding *Bd* – the geographic identification of the panzootic source.

Loci associated with lineage divergence

Divergence between the *Bd*-GPL and *Bd*-Brazil lineages is deep, as further evidenced by high F_{ST} between lineages. We identify blocks of the *Bd* genome contributing most to this divergence (Figure 3-3), and provide candidate loci that may be driving it (Figure 3-4). Some of the functional gene categories we identified in these divergence blocks correspond to gene functions (e.g. protein kinase activity among others) enriched in genomic regions previously implicated in *Bd* pathogenesis (Joneson *et al* 2011, Rosenblum *et al* 2013), found to be

upregulated when cultured with host tissue (Rosenblum *et al* 2012), and upregulated in live infections (Ellison *et al* 2017). The other functional category driving lineage differences draeing our interest was the genes related to managing oxidative stress (superoxide dismutases and oxidoreductases), also identified by some of these previous authors.

Functional studies are now needed to link the candidate loci we've identified to phenotypic variance, especially for traits related to virulence and transmissibility. The gene categories we describe, along with our field observations point to questions worth prioritizing in such studies. We know from prior studies that the temperature tolerance of *Bd* is low (only up to ~ 28º C; Johnson *et al* 2003; Retallick & Miera 2007; Woodhams *et al* 2012), and that habitats with intact canopy cover are favorable to *Bd* in the wild (Becker & Zamudio 2011). Our field observations show that *Bd*-GPL is capable of occupying habitats predicted to be least suitable for the species based on these and several other bioclimatic variables (James *et al* 2015). Our observations argue for investigation into how variants at these candidate genes – especially those acting on oxidative stress – confer advantage at extreme environments. This reverse ecology approach to identify genomic targets underlying ecological divergence has been applied to detect loci regulating heat tolerance in *Neurospora crassa* (Ellison *et al* 2011), and halotolerance in *Suillus brevipes* (Branco *et al* 2015). Our knowledge about genomic variation in *Bd* is continually becoming more refined, and now our understanding of phenotypic variation among *Bd* lineages must catch up if we aim to pinpoint the genes and genetics underlying its adaptive fitness in varying ecological contexts.

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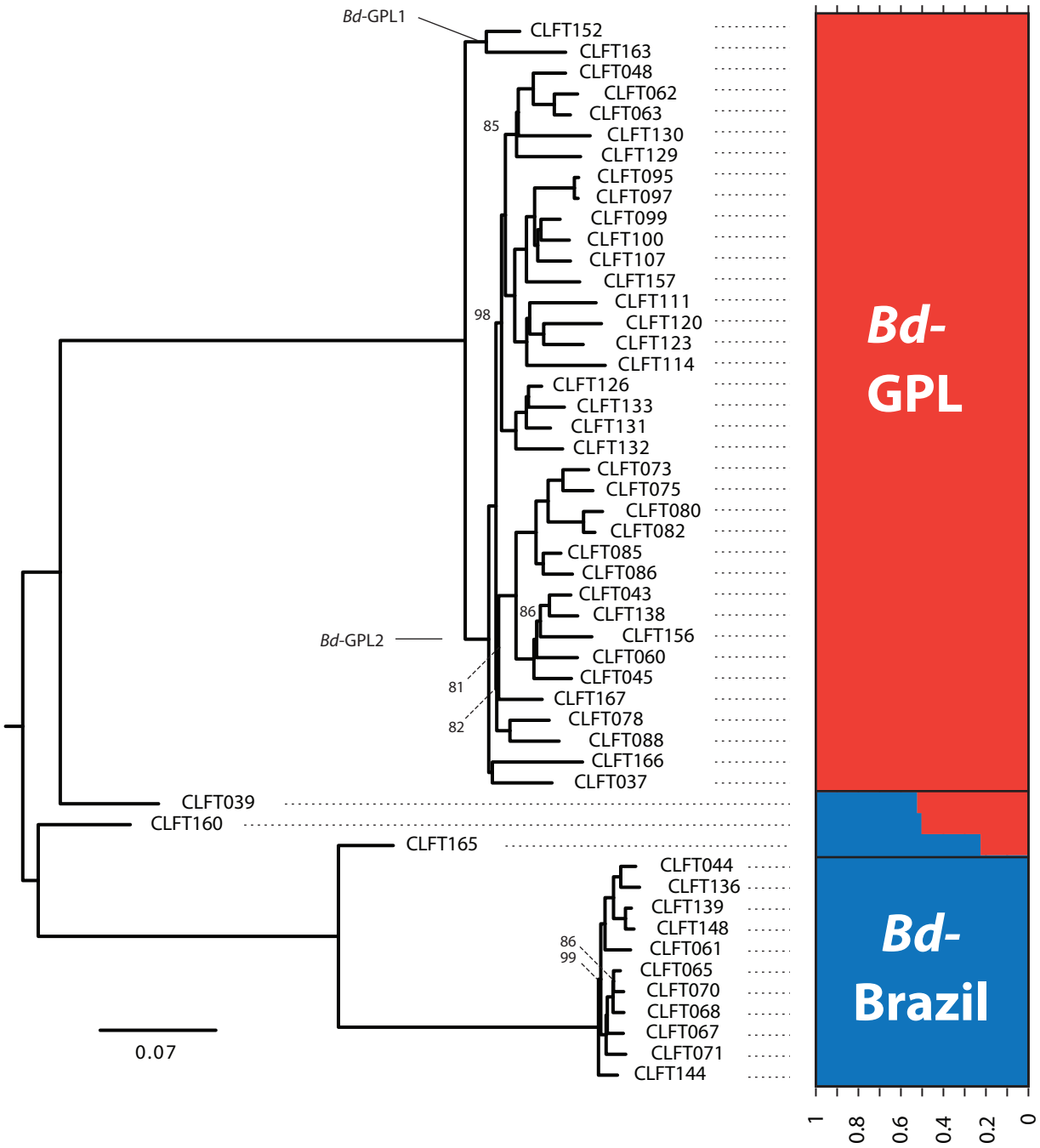

Figure 3-1: Neighbor joining dendrogram for 51 Brazilian Atlantic Forest isolates. Midpoint-rooted and based on genetic distance between 90,429 genomic SNPs. All branches are supported by 100% bootstrap support unless otherwise indicated. To the right is the associated structure plot showing the probability of ancestry to one of K = 2 lineages, *Bd*-GPL (red) and *Bd*-Brazil (blue). F1 hybrids show approximately 50% probability of ancestry to either parent, and the F2 hybrid CLFT165 shows approximately 75% and 25%. The nodes corresponding to previously described *Bd*-GPL subclades *Bd*-GPL1 and *Bd*-GPL2 are indicated.

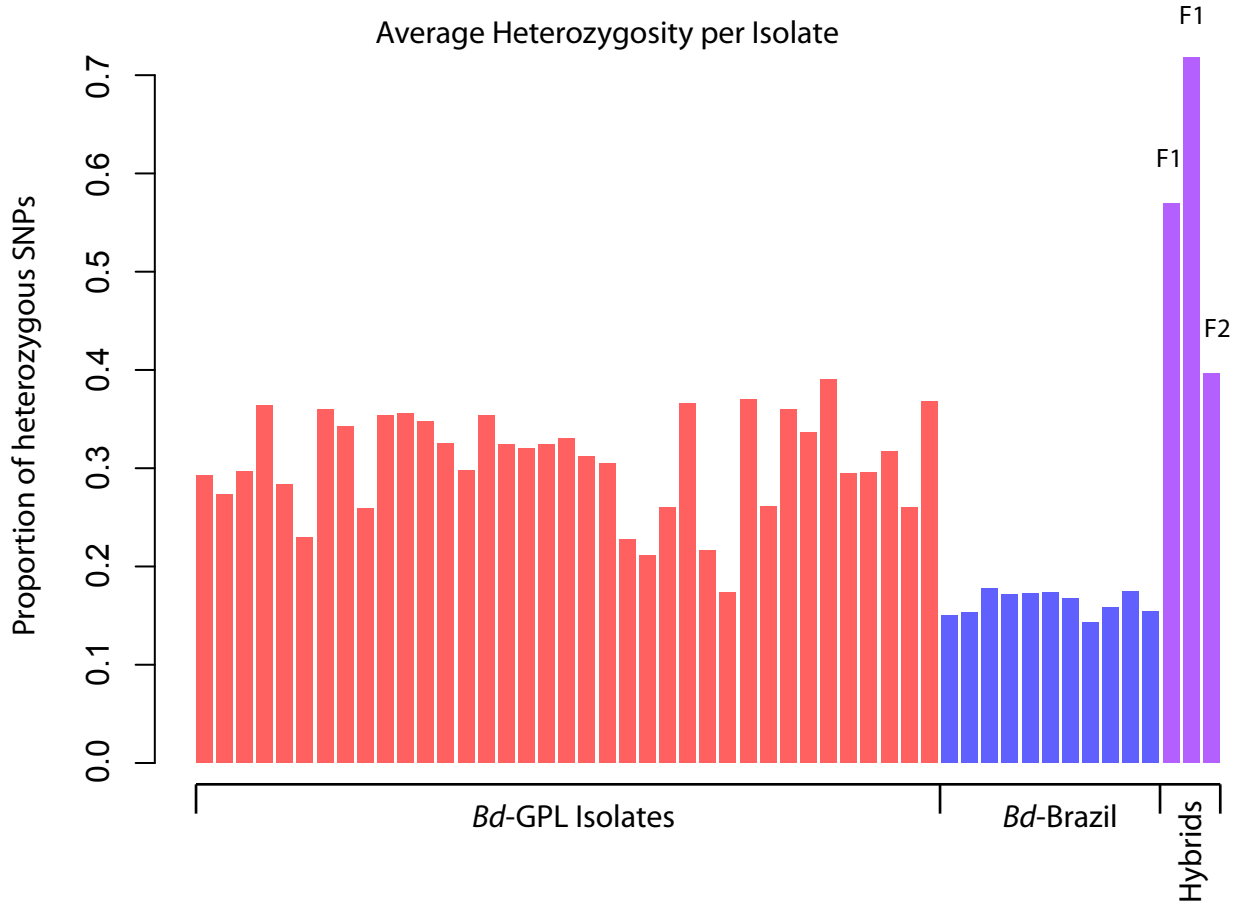

Figure 3-2: Barplots of average heterozygosity for each *Bd* isolate sequenced. Heterozygosity estimates presented here were corrected for sequencing depth. *Bd*-GPL isolates had a significantly higher proportion of SNPs with heterozygosity (Welch's $t = 15.48$, $p < 0.001$). Hybrid strains show the highest heterozygosity (rank:

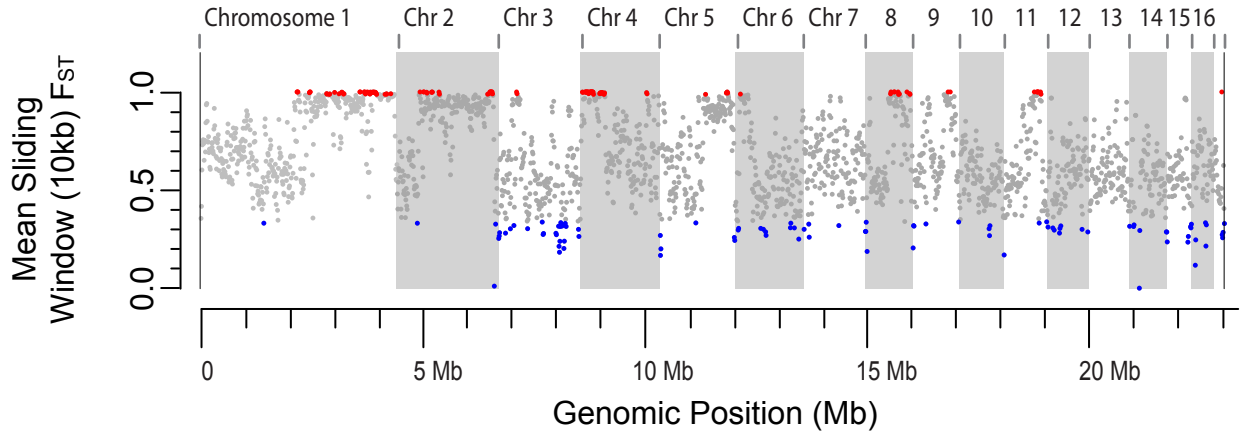

Figure 3-3: Plot of F_{ST} values between *Bd*-GPL and *Bd*-Brazil populations. Analysis of 10kb windows across the genome, the 5% of windows with highest F_{ST} used in the GO analysis (Figure 3-4) are highlighted in red. Divergent regions tend to occur in blocks along the genome, and some correspond to regions of shared LOH between isolates. The 5% of windows with the lowest F_{ST} are also shown for comparison (blue).

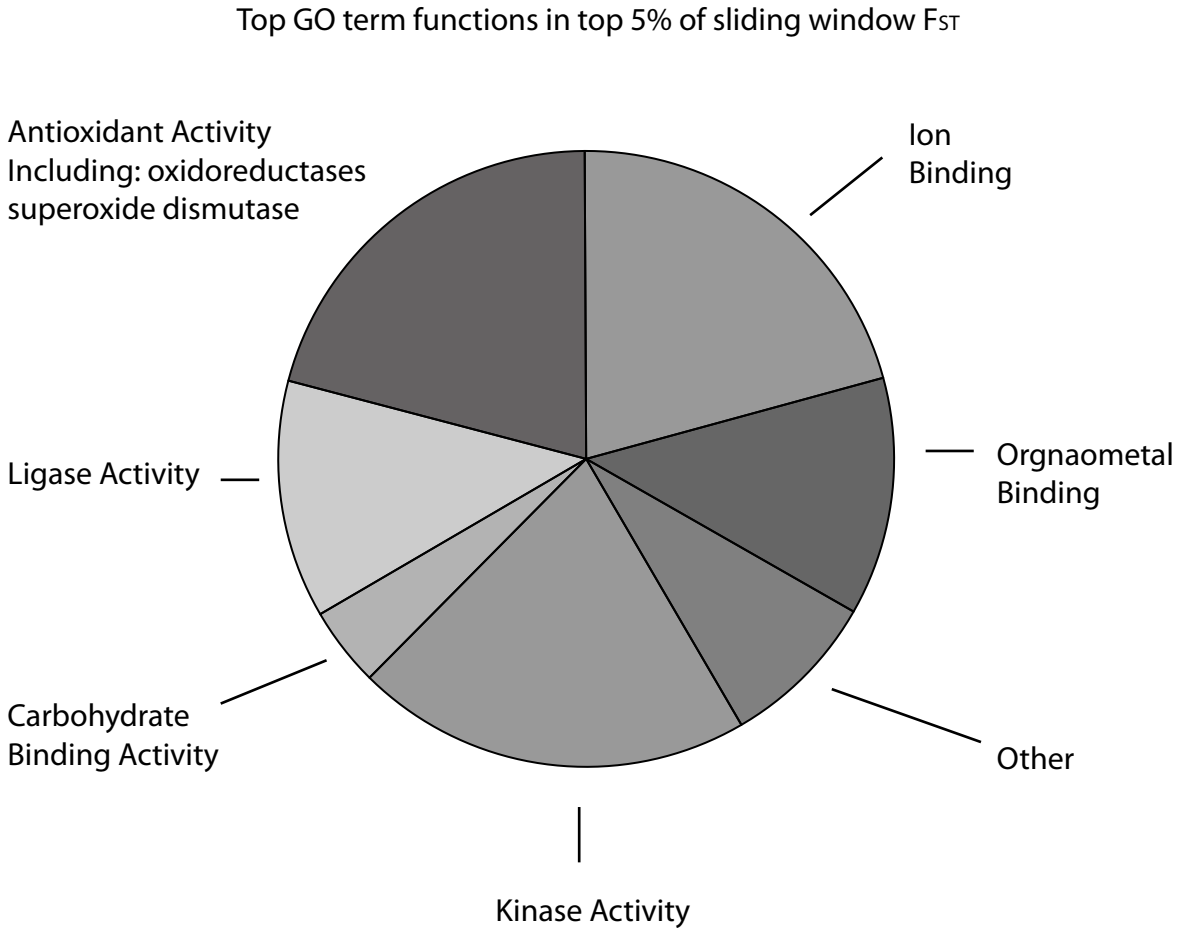

Figure 3-4: Pie chart of enriched gene ontology (GO) functional groups. Functional enrichment in highly diverged F_{ST} windows (Figure 3-3) (Full results of the GO functional analysis are presented in Table B-2).

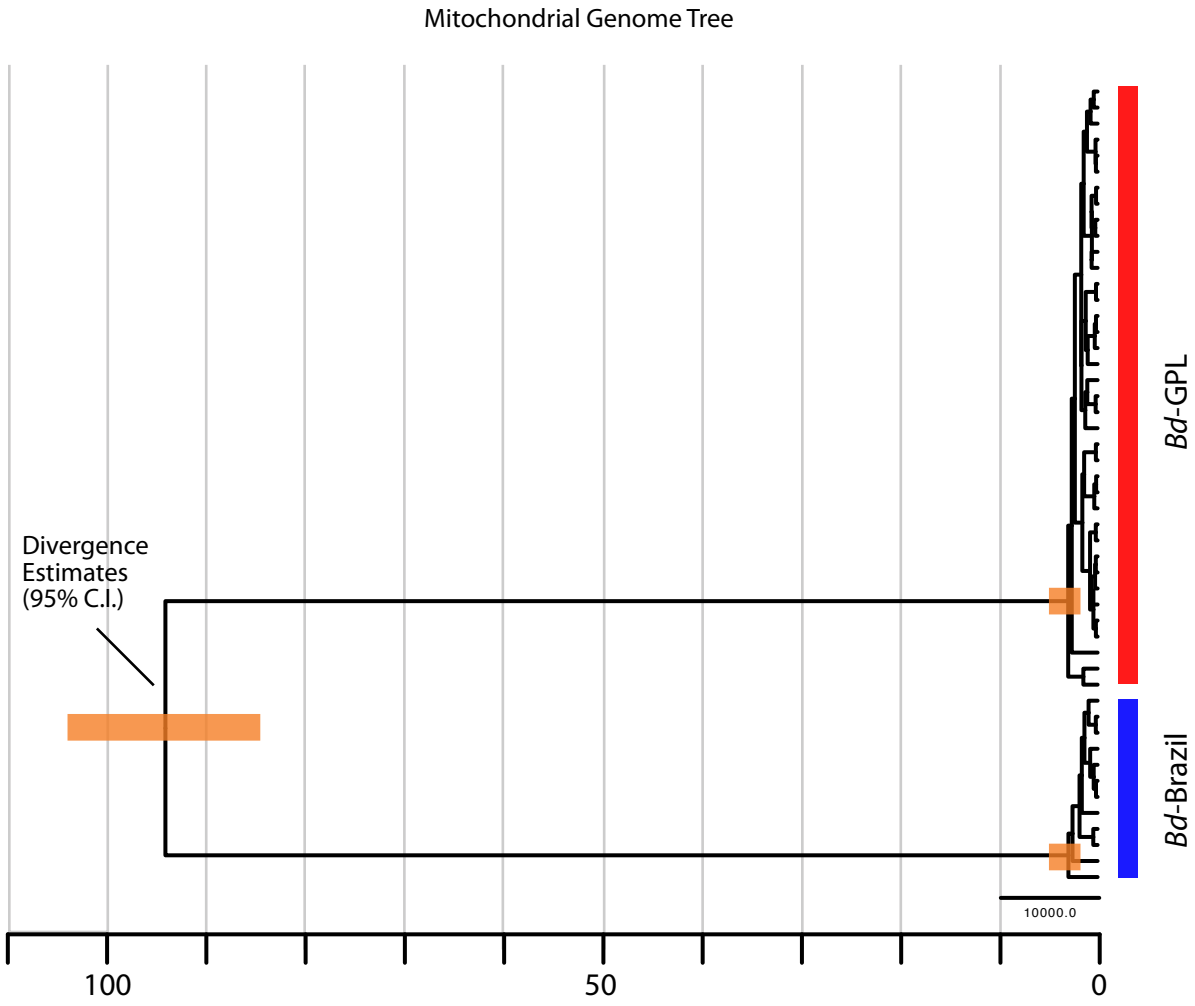

Figure 3-5: Time calibrated, ML phylogeny of the *Bd* mitochondrial genomes. Genomes of hybrids are uniparentally inherited. Orange bars show the 95% confidence interval around Bayesian divergence time estimates obtained using BEAST. Divergence estimates are shown for the entire tree, and for each major lineage found in the Atlantic Forest.

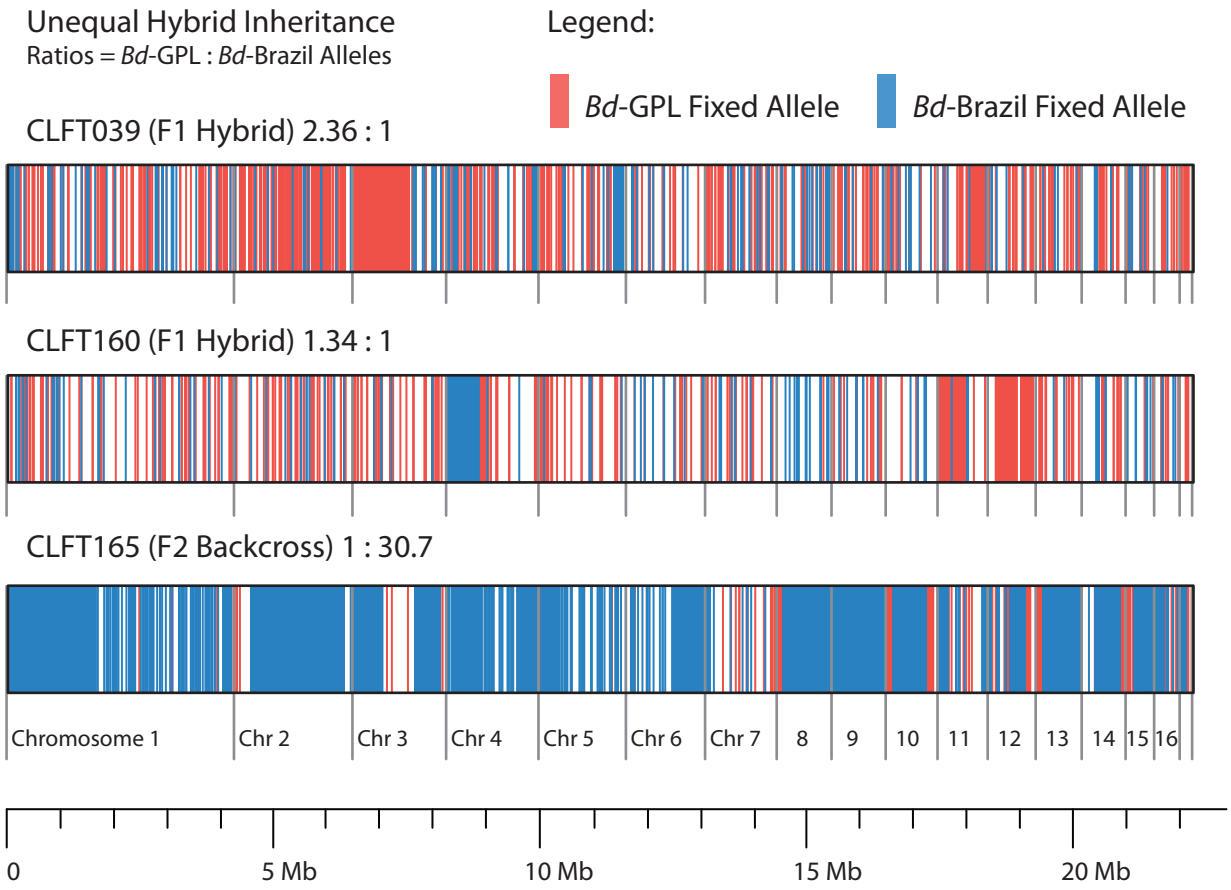

Figure 3-6: Patterns of unequal allele inheritance in hybrid strains. Positions of the hybrid *Bd* genomes homozygous for either parental allele are plotted in red (homozygous *Bd*-GPL allele) and blue (homozygous *Bd*-Brazil allele). Unequal inheritances from the reciprocally fixed SNPs favored the *Bd*-GPL parental allele in both F1 hybrids, and favored the backcross in the F2 hybrid. Ratios following the isolate codes indicate the ratio of unequally inherited *Bd*-GPL alleles to *Bd*-Brazil alleles.

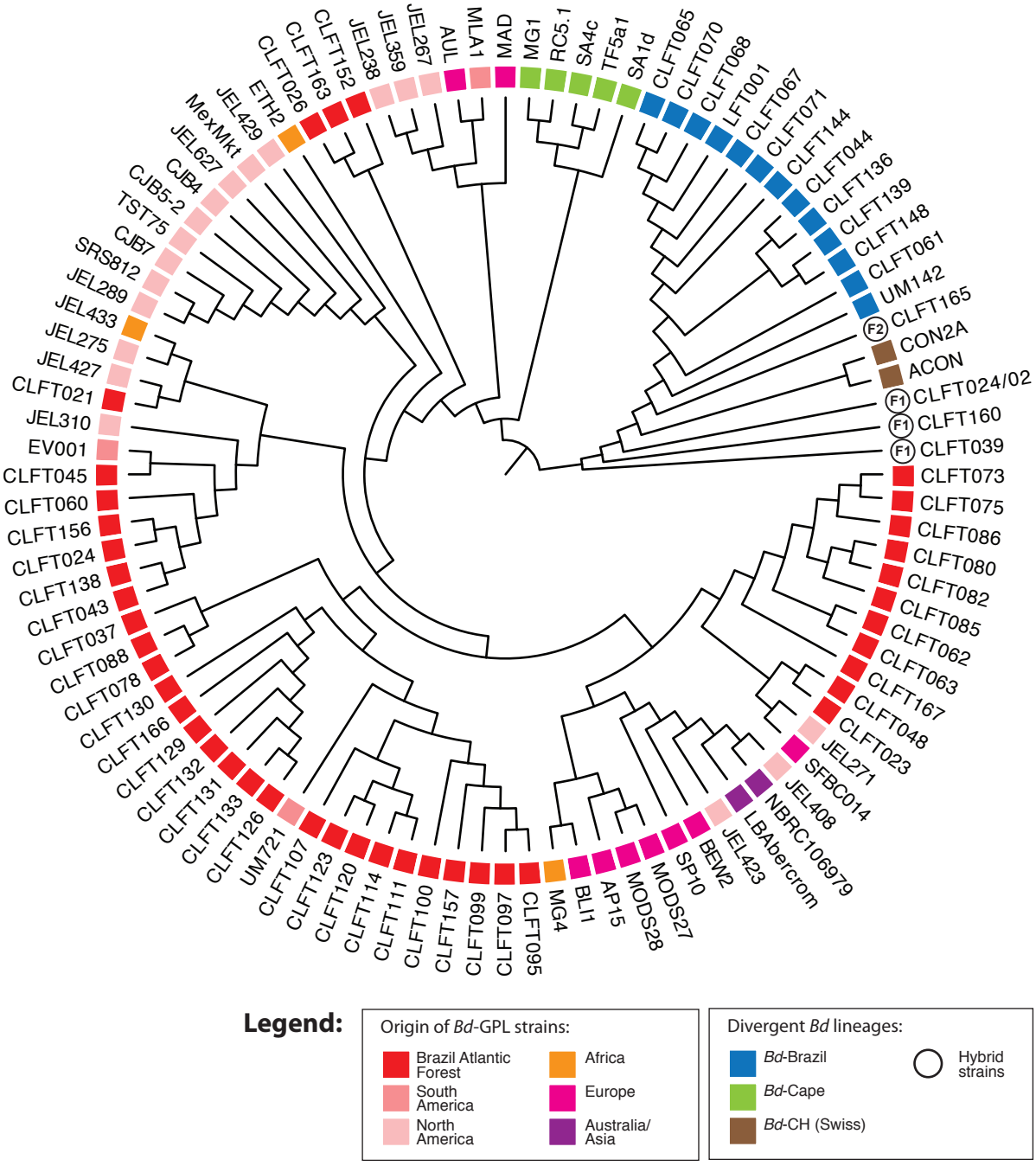

Figure 3-7: Meta-analysis of *Bd* genomes from a global panel of isolates. Neighbor-joining cladogram based on genomic SNPs called from newly sequenced *Bd* genomes from this study and previously published genome sequences from Farrer *et al* 2013 and Rosenblum *et al* 2013. Colors in the *Bd*-GPL clade indicate the geographic origin of each isolate.

Tables:

Table 3-1: Atlantic Forest *Bd* isolates used in genome sequencing study; with lineage assignment, collection data, and sequencing coverage

Isolate	Lineage	Geographic Origin, Municipality	State	Sequenced Depth	Aligned Depth
CLFT037	*Bd*-GPL	Reserva Betary, Iporanga	SP	29.63x	17.90x
CLFT039	F1 Hybrid	Serra da Graciosa, Morretes	PR	30.08x	16.16x
CLFT043	*Bd*-GPL	Serra da Graciosa, Morretes	PR	67.97x	29.12x
CLFT044	*Bd*-Brazil	Serra da Graciosa, Morretes	PR	61.27x	34.07x
CLFT045	*Bd*-GPL	Serra da Graciosa, Morretes	PR	67.78x	33.49x
CLFT048	*Bd*-GPL	Rancho Queimado	SC	56.20x	22.36x
CLFT060	*Bd*-GPL	Pomerode	SC	49.09x	27.39x
CLFT061	*Bd*-Brazil	Pomerode	SC	44.96x	25.55x
CLFT062	*Bd*-GPL	Pomerode	SC	43.98x	21.97x
CLFT063	*Bd*-GPL	Pomerode	SC	86.91x	34.75x
CLFT065	*Bd*-Brazil	Serra do Japi, Jundiaí	SP	113.07x	53.12x
CLFT067	*Bd*-Brazil	Serra do Japi, Jundiaí	SP	97.53x	44.51x
CLFT068	*Bd*-Brazil	Serra do Japi, Jundiaí	SP	63.01x	30.34x
CLFT070	*Bd*-Brazil	Serra do Japi, Jundiaí	SP	73.74x	34.48x
CLFT071	*Bd*-Brazil	Serra do Japi, Jundiaí	SP	71.25x	32.81x
CLFT073	*Bd*-GPL	Serra dos Órgãos National Park	RJ	44.50x	20.49x
CLF 075	*Bd*-GPL	Serra dos Órgãos National Park	RJ	64.87x	28.78x
CLF 078	*Bd*-GPL	Serra dos Órgãos National Park	RJ	89.26x	39.43x
CLFT080	*Bd*-GPL	Serra dos Órgãos National Park	RJ	80.67x	33.25x
CLFT082	*Bd*-GPL	Serra dos Órgãos National Park	RJ	41.09x	16.49x
CLFT085	*Bd*-GPL	Serra dos Órgãos National Park	RJ	62.11x	27.97x
CLFT086	*Bd*-GPL	Serra dos Órgãos National Park	RJ	54.42x	28.86x
CLFT088	*Bd*-GPL	Lago Iacy, Teresópolis	RJ	38.71x	17.86x
CLFT095	*Bd*-GPL	Serra Bonita, Camacan	BA	65.87x	34.08x
CLFT097	*Bd*-GPL	Serra Bonita, Camacan	BA	49.82x	25.64x
CLFT099	*Bd*-GPL	Serra Bonita, Camacan	BA	39.60x	20.81x
CLFT100	*Bd*-GPL	Serra Bonita, Camacan	BA	45.09x	22.85x
CLFT107	*Bd*-GPL	Serra Bonita, Camacan	BA	52.86x	21.70x
CLFT111	*Bd*-GPL	Santa Teresa	ES	52.80x	26.30x
CLFT114	*Bd*-GPL	Santa Teresa	ES	38.15x	19.14x
CLFT120	*Bd*-GPL	Santa Teresa	ES	40.40x	16.63x
CLFT123	*Bd*-GPL	Santa Teresa	ES	58.27x	15.72x
CLFT126	*Bd*-GPL	Vargem Alta	ES	47.85x	19.21x
CLFT129	*Bd*-GPL	Vargem Alta	ES	37.08x	16.08x
CLFT130	*Bd*-GPL	Vargem Alta	ES	37.34x	16.06x
CLFT131	*Bd*-GPL	Vargem Alta	ES	54.11x	27.72x
CLFT132	*Bd*-GPL	Vargem Alta	ES	46.15x	18.18x
CLFT133	*Bd*-GPL	Vargem Alta	ES	71.13x	30.99x
CLFT136	*Bd*-Brazil	Serra da Graciosa, Morretes	PR	64.31x	32.96x

CLFT138	*Bd*-GPL	Serra da Graciosa, Morretes	PR	47.81x	24.26x
CLFT139	*Bd*-Brazil	Serra da Graciosa, Morretes	PR	69.68x	35.20x
CLFT144	*Bd*-Brazil	Serra da Graciosa, Morretes	PR	60.85x	27.41x
CLFT148	*Bd*-Brazil	Serra da Graciosa, Morretes	PR	51.11x	25.43x
CLFT152	*Bd*-GPL-1	Serra da Graciosa, Morretes	PR	57.08x	26.97x
CLFT156	*Bd*-GPL	Serra da Graciosa, Morretes	PR	61.85x	28.49x
CLFT157	*Bd*-GPL	Serra da Graciosa, Morretes	PR	46.88x	22.82x
CLFT160	F1 Hybrid	Serra da Graciosa, Morretes	PR	57.22x	26.10x
CLFT163	*Bd*-GPL-1	Serra da Graciosa, Morretes	PR	40.07x	25.67x
CLFT165	F2 Hybrid	Serra da Graciosa, Morretes	PR	44.29x	23.35x
CLFT166	*Bd*-GPL	Campos do Jordão	MG	52.14x	24.39x
CLFT167	*Bd*-GPL	Campos do Jordão	MG	54.20x	23.41x

Brazilian state abbreviations are: Bahia (BA), Espírito Santo (ES), Rio de Janeiro (RJ), Minas Gerais (MG) São Paulo (SP), Paraná (PR), and Santa Catarina (SC).

Chapter 4

Intraspecific competition between *Batrachochytrium dendrobatidis* strains on a model amphibian host

Abstract:

Competition between pathogen strains may be a key driver of evolution in pathogens, particularly following a geographic invasion. Ecological theory predicts that direct competition between closely related disease strains in overlapping hosts will result in only the most virulent strain persisting. Despite its evolutionary implications, the outcome of competition remains untested for the vast majority of pathogens. Here, we experimentally investigate the *in-vivo* competitive differences between two highly divergent lineages of the amphibian-killing chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*). These divergent *Bd* lineages are hypothesized to have diverged in allopatry but recently been brought back into secondary contact by human introduction. Prior studies suggest that a panzootically-distributed, global lineage of *Bd* was recently introduced into southern Brazil, and is competitively excluding endemic lineages in the southern Atlantic Forest. To test for differences in competitive fitness, we performed coinfection studies between invasive versus endemic Brazilian *Bd* strains on a model host frog (*Hymenochirus curtiitpes*). We tracked zoospore production by strain over the course of the coinfection experiment with a chip-based digital PCR (dPCR) assay. We show that the globally invasive panzootic lineage has a competitive advantage in spore production especially during the first 1-4 weeks of infection, and on frogs succumbing to *Bd* infection. We also describe the

application of a novel technology (dPCR) for quantifying rare, strain-specific alleles within a mixed *Bd* population. Our study provides new evidence that competitive pressure resulting from the human movement of pathogens can be a potential force shaping the genetics and spatial epidemiology of pathogens in the wild.

Introduction:

Emerging pathogens are one of the greatest modern threats to global biodiversity, food security, and human health (Smith *et al*. 2009; Jones *et al*. 2008). Emergence could be linked with particularly virulent pathogen genotypes, lack of adaptive coevolution following genotype invasion, or hybridization. Competition between pathogen strains may also be a key driver of virulence evolution and disease emergence (Alizon *et al*. 2013, Zhan & McDonald 2013). This experimental study examines the competitive interactions between two highly divergent lineages of an emerging fungal pathogen under competition on live amphibian hosts. The competitive exclusion principle predicts that two competing lineages cannot occupy the same ecological niche indefinitely (Levin 1970; May 1975). Applying this principle to disease dynamics predicts that divergent pathogen strains will not stably coexist in the same host environment (Bremermann & Thieme 1989; Nowak & May 1994).

Epidemiological models support this idea, suggesting that competing pathogen strains in coinfected hosts will be under selection for faster growth and higher virulence, despite an accompanying tradeoff in increased host mortality (Anderson & May 1982; Levin & Pimentel 1981; Bremermann and Pickering 1983). This “shortsighted evolution” scenario leads to the competitive exclusion of all but the most virulent disease strains (Levin & Bull 1994). While this

hypothesis has limited empirical support in a few well-studied systems (*e.g.* malaria; de Roode *et* al. 2005a; de Roode *et al*. 2005b; Bell *et al*. 2006), it remains untested for the vast majority of pathogens. The possibility of selection for hypervirulence as a byproduct of the competition holds implications for the health of global ecosystems. Our study strains – isolated from a rare contact zone between lineages – provide a novel system to test ecological outcomes predicted by pathogen competition/exclusion theory. The implications of the study will help predict how landscape-level population genetics may shift in pathogen systems under competition. Understanding the ecological and evolutionary outcomes of pathogen strain competition is more critical than ever with the increasing global movement of pathogens by human activity.

Geographic distributions of Brazilian Bd strains

Chytridiomycosis of amphibians is the emerging infectious disease implicated in massive population declines and extinctions worldwide (Berger *et al.* 1998; Lips *et al.* 2006; Rachowicz *et al.* 2006). After appearing enigmatically in the late 20th century, chytridiomycosis quickly became one of the most significant threats to global amphibian biodiversity (Skerratt *et al.* 2007). The disease is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (hereafter *Bd*, Longcore *et al.* 1999). In many regions of the globe *Bd* is newly arrived and actively spreading. In other regions, however, *Bd* appears to be endemic and in stable equilibrium with its associated hosts. Evidence suggests that in regions experiencing dramatic amphibian declines, *Bd* has been recently introduced to naïve host populations (Morgan *et al.* 2007; Vredenburg *et al.* 2010; Cheng *et al.* 2011).

Our prior investigations showed that two deeply divergent lineages of *Bd* are found co-occurring in a narrow contact zone in the Atlantic Forest region of Brazil. The globally distributed, panzootic clone, termed *Bd*-GPL (*Global Panzootic Lineage*, Farrer *et al.* 2011), and a putative endemic lineage, *Bd*-Brazil (Schloegel et al, 2012), occupy overlapping ranges in this region. The geographic distributions of these lineages in the Brazilian Atlantic Forest show that the pandemically distributed *Bd*-GPL occupies most of the surveyed habitat (Jenkinson *et al.* 2016). Furthermore, *Bd*-GPL occupies more of the inhospitable extremes of this habitat as predicted by *Bd* habitat suitability models (James *et al.* 2015). These factors suggest that *Bd*-GPL may be excluding a less competitive *Bd*-Brazil lineage from all but the most suitable habitat. We use two *Bd*-GPL, and two *Bd*-Brazil strains isolated from field surveys of the Brazilian Atlantic Forest to test if intraspecific competition occurs between strains under laboratory conditions.

Evidence of strain coinfection in amphibians

The understanding that divergent *Bd* strains can coinfect individual hosts is relatively new. In Rosenblum *et al.* (2013) the authors whole-genome sequenced two strains (one *Bd*-GPL, CLFT024; and one *Bd*-GPL x *Bd*-Brazil hybrid, CLFT024_02) isolated from the same *Hylodes cardosoi* individual (Family: Hylodidae). We have also made an unpublished observation of multi-strain *Bd* coinfection in captive frogs. Since the discovery of the *Bd*-Brazil strain in a live North American bullfrog (*Lithobates catesbeianus*) for sale in a grocery market in Ypsilanti, Michigan (Schloegel *et al.* 2012), our research group has continued to monitor the infection status of the live bullfrogs for sale at this market. In the summer of 2012, we isolated both *Bd*-GPL and *Bd*-Brazil from a single individual market bullfrog at this location. We determined the

lineage identity of these isolates with a multilocus sequence typing approach described in Chapter 2.

What is known about lineage-specific virulence?

In addition to the globally dispersed *Bd* lineage responsible for major chytridiomycosis outbreaks (*Bd*-GPL, the *Global Panzootic Lineage*; Farrer *et al.* 2011), at least four previously unknown lineages of *Bd* have been newly described since 2011. These previously unknown lineages are: a putative African endemic (*Bd*-CAPE; Farrer *et al.* 2011), a putative European endemic (*Bd*-CH; Farrer *et al.* 2011), a putative Brazilian endemic (*Bd*-Brazil; Schloegel *et al.* 2012), and a putative Asian endemic (*Bd*-Korea; Batallie *et al.* 2013). Only two studies have tested for differences in virulence among these newly described lineages. In the first study, *Bd*-GPL was shown to cause mortality faster than either *Bd*-CAPE or *Bd*-CH when inoculated separately on *Bufo bufo* (European toad) in controlled experiments (Farrer *et al.* 2011). In a later study, our collaborative group showed that some *Bd*-GPL strains – but not all – cause mortality faster than *Bd*-Brazil in *Lithobates sylvaticus* (North American wood frog) hosts (Becker *et al.* 2017). Other studies have shown variation in virulence between differences within a lineage (*Bd*-GPL; Berger *et al.* 2005; Retallick & Miera 2007; Fisher *et al.* 2009; Garner *et al.* 2009), with some of this variation attributable to culture storage and passage conditions in the laboratory (Langhammer *et al.* 2013; Refsnider *et al.* 2013). However, no studies so far have explored the population-level outcomes of direct competition between any of the *Bd* lineages.

Here, we report on the first intraspecific competition study between *Bd* strains in a live amphibian host. We attempted to address one aspect of the overarching hypothesis that *Bd*-GPL

is expanding its range in the Brazilian Atlantic Forest by competitively excluding *Bd*-Brazil. We investigated if such competitive differences between Atlantic Forest *Bd* isolates exist under controlled laboratory conditions. The specific goals of this study were to quantify the relative performance of *Bd*-GPL and *Bd*-Brazil strains when inoculated on the same limited host resource, and to investigate the development of a model *Bd* host system, suitable for standardizing future laboratory-based virulence studies. The results of our study shed light on strain competition and exclusion as an ecological force capable of shifting pathogen genotype frequencies in mixed populations, and carries wide ranging implications for understanding the consequences of human-mediated pathogen introduction to endemic systems.

Materials and Methods:

Experimental design:

To investigate if relative fitness differences exist between *Bd* strains under competition, we tracked the population genetics of *Bd*-GPL x *Bd*-Brazil coinfections in 20 experimental amphibian populations. We measured differences in infective zoospore production at four time points over the course of a 10-week experiment. We employed digital PCR technology to provide absolute quantification of a newly developed mitochondrial marker designed to distinguish *Bd*-GPL from *Bd*-Brazil in a mixed sample (Bdmt26360; Rodriguez *et al. unpublished*).

We used the fully aquatic, dwarf clawed frog (*Hymenochirus curtipes*, Family: Pipidae) as the host species for this study. *H. curtipes* is tolerant of *Bd* infection at low levels, but succumbs to disease at high pathogen loads (Raverty & Reynolds 2001; Murphy *et al.* 2015). *H.*

curtipes is globally available from commercial suppliers in the aquarium trade, allowing for the replication of our investigation by other research groups, followed by the possibility for wider comparisons of *Bd* dynamics in a standardized model host. Adult dwarf clawed frogs range from 2.0 - 3.1 cm (snout vent length, SVL; Bartlett & Bartlett 1996) making the management of experimental colonies more feasible than for the much larger amphibian model species – the African clawed frog (*Xenopus laevis*, up to 12.7 cm SVL; also Family: Pipidae). Finally, *H. curtipes* are completely aquatic, surfacing only briefly to breathe, and do well housed in social groups (Bartlett & Bartlett 1996). This quality makes the species an attractive choice because we were interested in studying competition under a scenario of active strain transmission within a host population.

We selected two *Bd*-GPL isolates, and two *Bd*-Brazil isolates to compete in four possible combinations between the represented lineages (Figure 4-1a). This design addresses competitive differences between *Bd*-GPL and *Bd*-Brazil while accounting for possible strain-to-strain differences within each lineage, and differences in passage history between collection years. These isolates were all recently collected from the Atlantic Forest contact zones described in Chapter 2 and Jenkinson *et al.* (2016). We took care to passage the experimental strains minimally in culture (Table 4-1) to prevent major genetic or phenotypic changes under laboratory culture (Langhammer *et al.* 2103; Refsnider *et al.* 2013). Because each *Bd*-GPL and *Bd*-Brazil pair was collected within weeks of each other, each pair functions as a control for length of passage in culture. The first *Bd*-GPL and *Bd*-Brazil strains were collected from the northern Atlantic Forest contact zone in 2013 (isolated from an *Aplastodiscus sp*. in the state of Rio de Janeiro and from *Hylodes japi* in the state of São Paulo respectively). The second *Bd*-

GPL and *Bd*-Brazil strains were both isolated from *Hylodes cardosoii* individuals within 0.25 km of each other in 2014 in the Serra da Gracisosa, Paraná hybrid zone (Figure 4-1b).

Having been recently collected from Brazil, these *Bd* strains should not have been recently exposed to the *H. curtipes* host native to the Congo basin of central Africa. As such, at least some of these *Bd* strains will not have encountered a similar host environment in their recent ecological histories. This "common garden" set up will be a competitive landscape distinct from the host diversity available to either stain within their current ranges in the Atlantic Forest. Our experimental units consisted of 20 aquarium tanks containing small populations (n = 5) of dwarf clawed frogs. Each experimental population was randomly assigned to one of the four competition treatments described above (Table 4-2). Each of these four treatments was replicated in five experimental tanks (n = 100 individual frogs total).

Animal care procedures:

We performed all investigations involving live vertebrate animals following protocols approved by the University of Michigan's Institutional Animal Care and Use Committee (IACUC protocols: PRO00005605 and PRO00007691). Experimental animals were housed at the University of Michigan Life Sciences Institute Animal Care Facilities, and the experiments were conducted in consultation with the veterinary team from the Unit for Laboratory Animal Medicine. Below is a summary of the animal care procedures developed and utilized for this study. A full standard operating procedure for the handling of experimental *H. curtipes* approved by the University of Michigan IACUC is provided as an appendix (Appendix D).

We obtained 100 adult *H. curitpes* frogs from Live Aquaria Inc. (Rhinelander, Wisconsin, U.S.A.; shipped from California, U.S.A.). We housed the frogs in 38 L glass tanks (25 x 30 x 50 cm; Figure C-1) at a density of 5 individuals per tank. We filled the tanks with approximately 35 L of deionized water treated with aquarium conditioner (0.025 µL/L; for nitrogen control; Prime, Seachem Inc.) and pH adjusted to 7 (± 0.5). The experimental tanks were maintained between 19 and 20° C on a 12:12 hour day/night cycle. We fed the animals three times per week with frozen bloodworms (*Glycera spp.*) or brine shrimp (*Artemia spp.*). Every seven days, we tested the water parameters (nitrite, nitrate, ammonia, chlorine, and pH) and performed 1/3-volume changes of the tank water. We equipped the tanks with internal aquarium filters (biological and chemical filtration), which were only used prior to inoculation with *Bd*. The biological filter was initiated (cycled) by adding 20 mL of a nitrifying bacterial suspension (*Nitrosomonas* and *Nitrospira* spp.; Safe Start, Tetra Inc.) to each tank. Just before inoculation, we turned off the filtration system to avoid disrupting infective zoospores.

Treatment of incoming Bd infection:

Prior studies have shown that commercially obtained *Hymenochirus* can be shipped from the supplier with pre-existing *Bd* infection (Raverty & Reynolds 2001; Murphy *et al.* 2015). Because of this, all incoming frogs were swabbed for a *Bd* infection assay by qPCR (following methods described below), and treated for prior *Bd* to the experiment. The initial qPCR assays showed that 18% of our 100 incoming animals were infected with relatively low spore loads of *Bd* (mean = 22.89 genomic equivalents per swab ± 6.68 S.E.M.). We cleared incoming infections by heat-treating all the animals prior to the experiment (Johnson *et al.* 2003; Retallick & Miera 2007; Woodhams *et al.* 2012). We slowly (5-7 days) raised the water temperature to 30-31° C and

maintained this high temperature for seven days. After the heat treatment, and a three-week recovery period, post-treatment qPCR assays were negative for all animals.

Inoculating Batrachochytrium dendrobatidis coinfections:

We conducted preliminary trials to determine a sub lethal dose of *Bd* for our experimental system. Our goal was not to induce or measure mortality as an effect, but rather to inoculate animals with an optimal dose of *Bd* that would result in the maintenance of infection over the study period to allow the observation the inter-strain competition dynamics over time. While our experimental dose did cause mortality in some of our animals, most populations survived and maintained the infection over the course of our experiment (details in 4.4 Results). Our initial trials were conducted with inoculation concentrations of $2x10^5$ zoospores/mL and $4x10^5$ zoospores/mL in 100ml spore baths which our trial animals (n = 8 individuals) cleared the infections within six weeks. Based on these trials we increased or final inoculum for the experimental treatments by almost one order of magnitude.

Prior to the experiment, we maintained *Bd* cultures in liquid 1% tryptone media (10 g tryptone, 10 g agar, 1 L H_20). Routine transfers were performed every 4 months, and the cultures were stored at 4º C between transfers. We propagated experimental *Bd* strains from actively growing liquid cultures on 1% tryptone agar plates (10 g tryptone, 10 g agar, 1 L H_2O) until sufficient growth had occurred for spore harvest (~6 days). We harvested *Bd* zoospores by flooding the agar culture plates with 3 mL of sterile water and collecting the suspended zoospores with a serological pipette. We quantified the concentration of zoospores in each spore suspension by counting zoospores (with intact flagella present) using a hemocytometer

(Neubauer, 0.1mm depth), and prepared standardized spore suspensions to batch inoculate groups of frogs (in 50 mL polypropylene tubes (Falcon, Corning Inc.). Each population (n = 5) was exposed to a 20 mL zoospore bath containing 10^7 infective zoospores from *each* of two competing strains (total exposure in 20 mL = $2x10^7$ zoospores; combined inoculum concentration = 10^6 zoospores/mL). The exposure baths were incubated at 19 -20° C with the 50 mL tubes placed horizontally for 6 hours. After the exposures, all animals were released back to their respective tanks and monitored daily for mortality or signs of morbidity.

Data collection:

We monitored infection progress for 70 days (10 weeks) post inoculation by swabbing the animals weekly with sterile skin swabs (MW 113, Medical Wire and Equipment Co.). We swabbed the inter-digital toe webbing of each foot 10 times and 10 times on both lateral surfaces of the abdomen (60 passes total). We employed the same procedure to swab any dead or moribund individuals encountered during daily health checks (n = 38). We extracted genomic DNA from skin swabs using 50 µL of PrepMan Ultra sample preparation reagent (Hyatt *et al.* 2007). For routine monitoring of *Bd* infection through the course of the experiment, we performed singlicate real-time quantitative PCR (qPCR) reactions (qPCR reaction conditions and cycling parameters in Table C-1) on a QuantStudio3 Real Time PCR System (Thermo Fisher Inc). Our qPCR zoospore standards ranged from 0.1 to 100,000 genomic equivalents (GE). The zoospore standards for qPCR were prepared using *Bd*-GPL strain CLFT035 collected in 2013 from São Paulo State, Brazil (Jenkinson *et al.* 2016). Quantification of qPCR results based on cycle thresholds from standard curve was performed with the QuantStudio Design and Analysis

software. We considered a qPCR quantification less than GE = 1 per swab to be negative for *Bd* infection.

To assess the population genetic outcome of strain competition, we chose four equally spaced temporal samples (weeks 1, 4, 7, and 10) for strain identification, and quantification using chip-based digital PCR (dPCR). We used the zoospore density (GE/μL) of each strain – quantified by dPCR – as our indicator of competitive fitness. The chip dPCR platform is a promising, new approach that achieves much greater precision in detecting rare allelic variation in a sample than traditionally performed, multiplexed qPCR assays (Conte *et al.* 2015; Salvi *et al.* 2015). Briefly, the QuantStudio 3D digital PCR system (Thermo Fisher Inc) partitions a duplexed, lineage-specific TaqMan assay into a PCR reaction chip (one sample per chip) made up of 20,000 nanowells (60 μm per well). We adjusted the sample dilution such that some wells receive target DNA, while others do not (appropriate dilutions for each sample were estimated using the associated qPCR quantification of total *Bd* DNA). Wells that received a target sequence, and amplified successfully in the PCR step released a specific fluorescent signal (probe sequences, dPCR reaction conditions, and cycling parameters in Table C-2). Post-PCR, we used the QuantStudio 3D digital PCR chip reader to detect the fluorescence signal of reporter dyes from each nanowell (from the VIC-tagged *Bd*-GPL probe, and FAM-tagged *Bd*-Brazil probe). The chip reader established an absolute count of successful, allele-specific amplification wells per chip. We then converted these counts to strain-specific measurements of zoospore density in our analyses.

Data Analyses:

We used the QuantStudio Cloud Analysis software to apply a count correction based on a Poisson distribution of probabilities that more than one target molecule from the reaction mix is partitioned into a single well. This correction yields the concentration of target sequence copies per μL of template solution. However, even closely related strains of *Bd* can vary in molecular marker copy number among individuals (Longo et al 2013; Rosenblum *et al.* 2013; Farrer *et al.* 2013). We addressed this by first determining the degree of copy number variation among strains for the mitochondrial assay marker (Bdmt26360). We made 10^6 zoospore standards for each of the four strains by counting zoospores with a hemocytometer as before. We extracted DNA from these zoospore standards using the methods described above, and performed the same dPCR assays on a serial dilution of the DNA extracts (10^3, 10^4, and 10^5). We constructed standard curves, and calculated the slope of the linear relationship between marker concentrations (copies/μL) and known dilutions. For each strain, we multiplied the slope of its dPCR standard curve by observed copy concentrations to determine zoospore density (GE/μL).

To improve the variance homogeneity in zoospore density across partitions (by lineage, by strain, by time point), we log transformed ($\log_{10}(x + 1)$) observed zoospore densities prior to analyses. Despite log transformation, the majority of our dPCR data partitions fail Bartlett's test for homogenous variances, and fail the Shapiro-Wilk test for distributions consistent with normality. Because of the non-normal distributions, and variance heterogeneity within these data, we did not perform parametric analyses of variance, opting instead for non-parametric methods in all downstream hypothesis testing. We performed all statistical tests in R v3.3.1 (R Core Team, 2016).

Results:

Bd infection over 10 weeks:

From week 1 to week 6 post-inoculation, mean infection loads (assessed by qPCR) plateau between 10^2 - 10^3 GE per swab. After the sixth week, we observe a rapid drop in infection loads continuing to the end of the experiment (Figure 4-2). Infection loads dropped by an overall rate of 311.5 GE per week (linear regression: slope = -311.5, $R^2 = 0.557$, $p = 0.008$). Many of the *H. curtipes* hosts repress (or fully recover from) *Bd* infection over the 10 week period, however, the individual host outcomes ranged widely. Of the 100 starting animals, 21% of individuals tested negative for *Bd* infection by the end of 10 weeks, while 38% of individuals died (or were euthanized because of disease signs). The time to death of succumbing individuals was not associated with any of the four competition treatments (Kruskal-Wallis rank-sum test, $\chi^2 = 0.63$, df = 3, $p = 0.889$).

Competitive effects between Bd-GPL and Bd-Brazil:

We partitioned the weekly zoospore densities by lineage to test for competitive differences in spore production. *Bd*-GPL produced higher spore densities than *Bd*-Brazil at all time points (Figure 4-3). We observed significant differences in zoospore density between lineages at certain time points (Kruskal-Wallis rank-sum test, $\chi^2 = 213.84$, df = 7, $p < 0.001$). We assessed pairwise differences between these lineages partitions *post-hoc* using Dunn's test of multiple comparisons with a Bonferroni correction (Table C-3). Our Dunn's test comparisons showed that *Bd*-GPL produced significantly more spores than *Bd*-Brazil early in the infection, during week 1 (corrected $p < 0.001$) and week 4 (corrected $p < 0.001$). By week 7, *Bd*-GPL spore densities are reduced from previous weeks, whereas mean *Bd*-Brazil spore density increased

from the previous time point. *Bd*-GPL still produced more spores on average than *Bd*-Brazil, but the differences in density are statistically indistinguishable (corrected p = 0.080). Finally, by week 10, spore density continued to drop for both strains. Again, mean *Bd*-GPL spore densities are greater than *Bd*-Brazil, but not significantly (corrected p = 0.370).

To visualize the magnitude and breadth of competitive differences between lineages at the level of individual hosts and experimental tanks, we plotted the difference between *Bd*-GPL and *Bd*-Brazil spore densities (Δ = (*Bd*-GPL GE/μL) - (*Bd*-Brazil GE/μL)) taken from each individual host (Figure 4-4). A positive delta indicates a greater density of *Bd*-GPL spores and negative deltas correspond to greater densities of *Bd*-Brazil spores. Overall, the calculated deltas were positive (mean week 1 Δ = 69.98, mean week 4 Δ = 93.65, mean week 7 Δ = 7.87, mean week 10 Δ = 11.84). We observed that competitive differences were greatest in the first part of the infection experiment (week 1 and 4), and grow weaker as the infection appears to be suppressed (week 7 and 10). This visualization of the data allowed us to see that variation in competitive spore production was largely shared across individuals in a tank. For example, all individuals assessed in Tank H during week 4 showed strong negative deltas suggesting that competitively superior strains on individuals are transmitting spores across individuals in the population.

Differences in competitive fitness among isolates

We also partitioned spore density data to test for competitive differences between our four individual *Bd* isolates. Within each lineage, we observed significantly superior and inferior competitor strains (Figure 4-5). Both CLFT137 (*Bd*-GPL) and CLFT150 (*Bd*-Brazil) were

significantly better competitors than their co-lineage counterparts CLFT073 (*Bd*-GPL) and CLFT070 (*Bd*-Brazil) (Kruskal-Wallis rank-sum test, $\chi^2 = 283.2$, df = 15, $p < 0.001$). We again assessed differences *a-posteriori* between isolate partitions using Dunn's test (Table C-4). In week 1, both *Bd*-GPL isolates and the competitively superior *Bd*-Brazil isolate produce significantly higher zoospore densities than the competitively inferior *Bd*-Brazil. In week 4, the competitively inferior *Bd*-GPL isolate (CLFT073) produced a lower density of spores than CLFT137, but this difference was not significant ($p > 0.05$). The average increase in *Bd*-Brazil spore density at week seven was driven entirely by the competitively superior *Bd*-Brazil isolate (CLFT150). It is at week 7 where the competitive differences among isolates are most pronounced. For both lineages, the competitively superior isolates (*Bd*-GPL: CLFT137, *Bd*-Brazil: CLFT150) produced significantly higher zoospore densities than their competitively inferior counterparts in the same lineage (*Bd*-GPL: CLFT073, *Bd*-Brazil: CLFT070). However, the competitively superior *Bd*-GPL isolate still produced higher average spore densities than the competitively superior *Bd*-Brazil isolate (although not significantly at $p > 0.05$). By week 10, the most competitive of the four isolates (CLFT137) produced higher average spore densities than the other isolates (not significantly; $p > 0.05$). The least competitive of the isolates (CLFT070) produced significantly lower spore densities than the other isolates at week 10.

Zoospore densities at host death

The zoospore densities from post-mortem skin swabs ranged an order of magnitude greater than those from live animals (live spore densities: ~0.0 - 10^3 GE/μL; post death: ~0.0 - 2.0×10^4 GE/μL). Because of this discrepancy in range, we analyzed zoospore densities from the post-mortem swabs separately from the other infection data presented above. *Bd*-GPL zoospore

densities were significantly higher than *Bd*-Brazil spore densities on dead individuals (Wilcoxon rank-sum test, $W = 1309$, $p < 0.001$; Figure 4-6). As with spore densities on live individuals, we observed differences in average spore density between the more and less competitive isolates, but they were not significant at $\alpha = 0.05$. A linear regression showed a slight decrease (slope = -0.184) in most-mortem *Bd*-GPL zoospore densities over the course of ten weeks ($R^2 = 0.140$, $p = 0.016$), and no temporal trend in post-mortem *Bd*-Brazil spore densities ($R^2 = 0.001$, $p = 0.854$).

Discussion:

We show for the first time that significant in-host competitive differences exist between major *Bd* lineages *in-vivo*. We also show that a hierarchical relationship of competitive fitness exists among individual isolate strains of *Bd*. As Read & Taylor (2001) point out, the evolutionary and epidemiological consequences of this in-host strain competition depend crucially on how competitive dynamics affect transmission to new hosts. Little is known about this critical step in the infection cycle for populations of genetically diverse pathogens. In the few infectious systems that have been studied – such as *Plasmodium chabaudi* (an apicomplexan causative agent of rodent malaria) – there is not clear connection between the potential for host damage (virulence) and the transmission potential required to maintain disease in a host population. In the example of *P. chabaudi* the dominance of a specific clone in a mixed infection paradoxically did not translate to increased transmission success of that clone perhaps due to evolutionary constraints on host exploitation versus transmission (Taylor *et al.* 1997).

In this study, however, our measures of competitive fitness – based on infective zoospore production – are directly tied to the mode of transmission for this pathogen (as opposed to

measures of host exploitation). This connection suggests that the competitive advantage we observe in the laboratory could translate to higher transmission success in the field, thereby resulting in rapid range increases of recently arrived strains. This ecological problem may be further compounded by prior observations that *Bd* strains reisolated from experimentally infected animals show increased virulence in subsequent exposures (Brem *et al.* 2013), and that increased host mortality (not just transmission) is correlated with *Bd* phenotypes showing greater rates of spore production (Langhammer *et al.* 2103). In contrast to the *P. chabaudi* system, *Bd* may have (at least temporarily) shed the theoretical constraints imposed by inherent tradeoffs between host exploitation and transmission (Alizon *et al.* 2009).

In addition to the competition between *Bd* lineages, each *Bd* strain is also in conflict with the host's innate immune function. 62% of our *Hymenochirus curtipes* hosts either repressed, or completely cleared *Bd* infections at the end of the experiment. This suggests that at least some of the competitive effects we observed were the result of "common gardens" that represented a harsh immunological landscape for *Bd*. As such these observations are relevant to inferring the outcomes of strain competition in novel hosts outside of the native strain ranges, which would be the case under a scenario of biological invasion.

Differences in competitive success between lineages

We observed significant differences in competitive success at the most critical phase of this infection process for transmission – the peak of zoospore production in the first 1-4 weeks. By the end of the experiment, when our hosts had largely cleared the infection, these competitive differences observed were no longer statistically significant. However, one must consider the

total zoospore densities at this stage when interpreting these results. Zoospore densities measured at the latest stage of our experimental infection/immunity process ranged from just ~0.0 - 195 GE/μL (mean = 13.38 GE/μL, median = 0.44 GE/μL). Factoring in the proportion of our whole DNA extract used as dPCR template, these densities translate to a median of only 22 zoospore equivalents in 60 swabbing passes across a 2.5cm long frog. While much remains unknown about the zoospore transmission thresholds in nature, spore densities this low may not be biologically relevant for transmission success in field settings, however, the experimental setup described here would allow for future testing of transmission rates by use of uninfected frogs.

Our measurements of post-mortem spore density from the 38% of host individuals succumbing to infection ranged to over an order of magnitude greater than those collected from seemingly healthy individuals. Because we held the inoculation dose of *Bd* and other experimental conditions constant across trials, we can only assume that the variation in host outcomes was due to host immune diversity at the individual or group level. While our aim in this study was not to assess host mortality induced by our study strains, the host deaths provided an opportunity to observe that competition results favored *Bd*-GPL regardless of host immuno-competence, but that the host composition affects the magnitude of *Bd* success. The significant increase in spore loads upon death suggests that the transmission outcomes of coinfection in nature will be dependent on host immunological diversity.

Within lineage differences in competitive success

Within each lineage, we observed one competitively superior and one competitively inferior isolate. At this point, it remains unclear whether these within lineage differences can be

ascribed to natural variation among *Bd* isolates. Prior research has shown in phenotypic variation even among closely related genetic strains of *Bd* collected within small geographic scales (Berger *et al.* 2005, Becker *et al.* 2017). We also now have a recent understanding that genomic changes and virulence attenuation can occur in *Bd* laboratory strains serially passage over long periods of time (Retallick & Miera 2007; Langhammer *et al.* 2013; Refsnider *et al.* 2015). These prior studies, however, documented changes in cultures passaged over 30 times in the span of six years. When our experimental inoculations took place, all experimental strains had been passaged 6-9 times. While we took measures to passage strains minimally, it is extremely difficult to obtain cultures for laboratory studies isolated from remote, international field sites with no passage history. Our study design controlled for passage history by selecting paired *Bd*-GPL and *Bd*-Brazil isolates collected contemporaneously, and passaged an equal number of times in culture. It is striking that the competitively inferior strains for each lineage were both passaged three more times that the competitively superior strains. While our study cannot identify whether this competitive performance is attributable to passage history, our results still showed that the *Bd*-GPL strain with 9 passages significantly outperformed its *Bd*-Brazil counterpart with 9 passages, and the same pattern held for the *Bd*-GPL and *Bd*-Brazil pair with 6 passages.

Novel methods for understanding Bd ecology and evolution

In addition to the ecological and evolutionary implications of our results, our study serves as a proof of concept for two important new tools to improve our understanding of this ecologically critical pathogen. First, we show that dPCR is a viable tool for the detection of rare *Bd* genotypes in mixed pathogen populations. This application shows potential for widespread

applications in the study of *Bd* from laboratory studies investigating *Bd* population dynamics to field surveys for pathogen genotypes. Because of its extreme sensitivity (the ability to detect *one* variant copy in 5 µL of sample), and its ability to simultaneously quantify and genotype samples, this dPCR assay is ideal for the detection of rare variants within a population – two problems frequently encountered in *Bd* studies. Our colleagues have now shown that the Bdmt26360 marker assay run on the dPCR system outperforms the same assay (primers, probes and fluorescent dyes) run as a traditional real-time qPCR reaction on known spore concentration standards from cultures (Rodriguez *et al. in prep*). Digital PCR outperformed both in the precision of quantification (especially at low loads), and accuracy of stain identification (because there is no allele drop out where the fluoresce signal of a rare allele is disrupted out by the overwhelming signal of a very common allele).

Finally, we describe a potential model in which to test *Bd* phenotype across a standardized host species. At present, virulence studies are typically conducted on haphazard assemblages of locally available species, which makes replication of experiments across research groups difficult. The *Hymenochirus* system offers a practical, economical, and globally available model host that would allow the standardization of virulence studies, should the *Bd* community adopt it. We have optimized the care protocol and describe a simple procedure to clear incoming animals of background *Bd* infection which we know to be a problem in amphibians associated with the aquarium trade (Raverty & Reynolds 2001; Murphy *et al.* 2015). We do not envision *Hymenochirus* to be the only host for future experimental infection studies, but rather a starting point for exploring *Bd*-amphibian interactions and a complement to studies involving locally available amphibian hosts.

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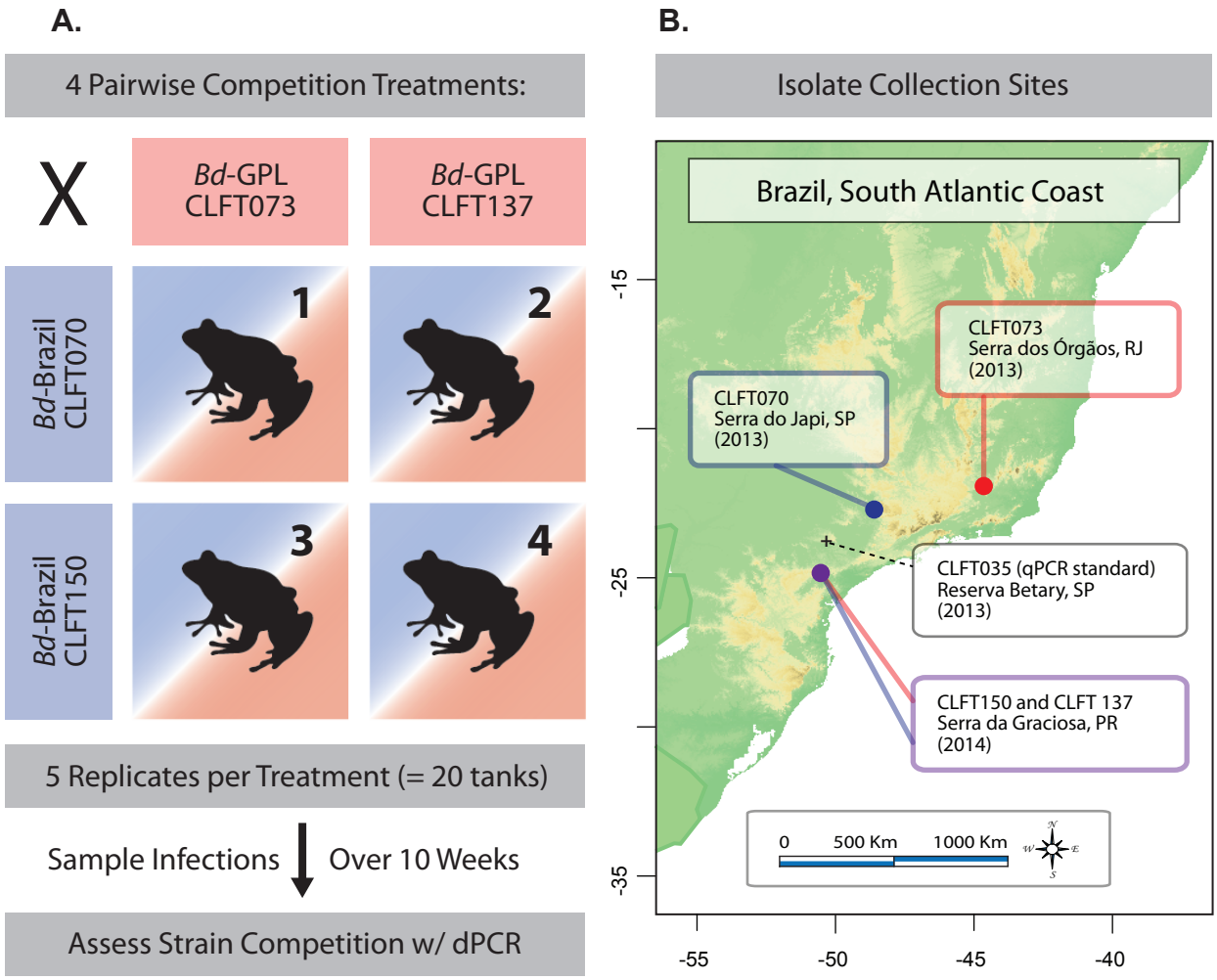


Figure 4-1: Experimental design of the pairwise strain competition experiment. Two *Bd*-GPL and two *Bd*-Brazil strains were competed in the four possible GPL x Brazil combinations. We repeated each combination 5 times for a total of 20 tanks (A). Collection localities, dates, and isolate codes for the strains examined in this study (B).

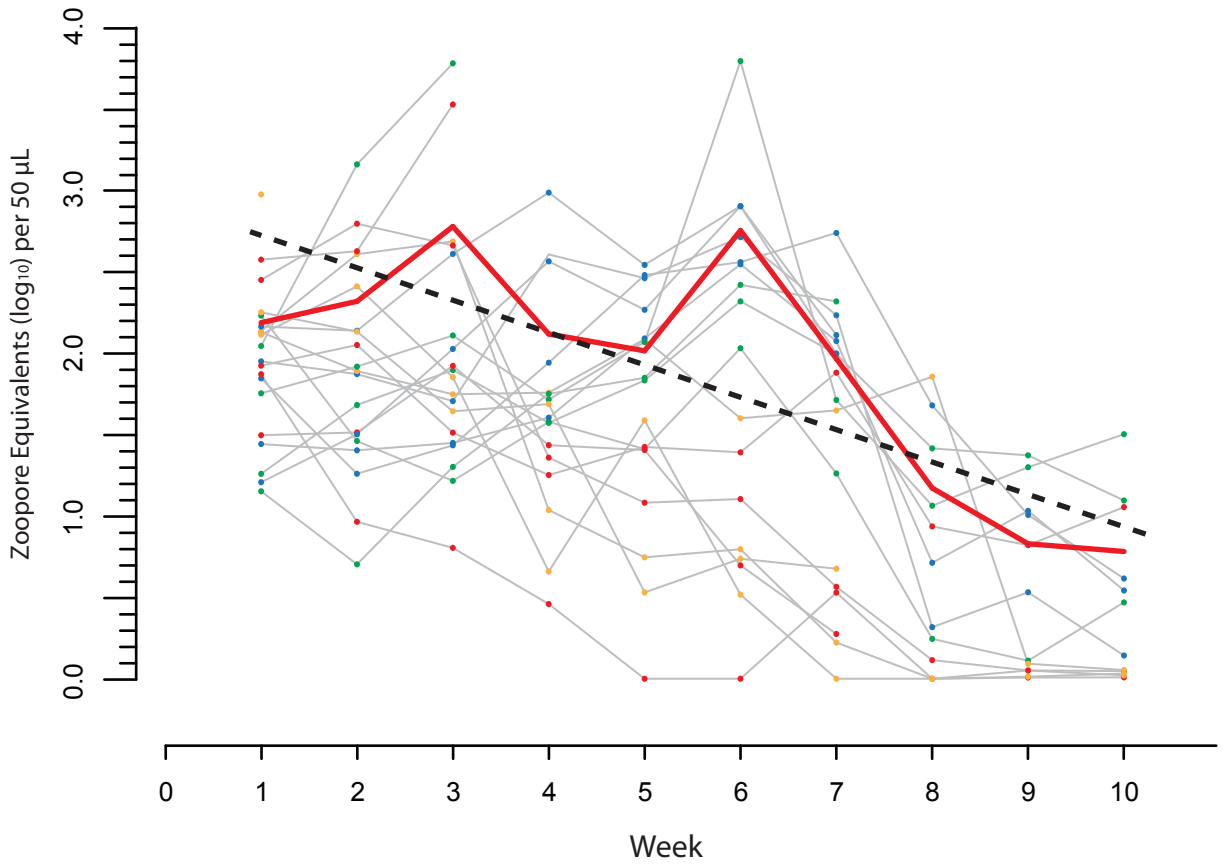


Figure 4-2: Infection dynamics and trend over the 10 week experiment. Red line shows the trend of weekly mean zoospore loads. Grey lines show the temporal dynamics of infection by individual tanks (n = 20), with colored dots representing one of four coinfection treatments (red: treatment 1, green: treatment 2, yellow: treatment 3, blue: treatment 4; see Table 4-2). Grey lines end at the point where all five animals in a tank have succumbed to disease. Dashed line showed a fitted linear model indicating an overall decrease in infection load over time (slope = -311.5, R^2 = 0.557, p = 0.008).

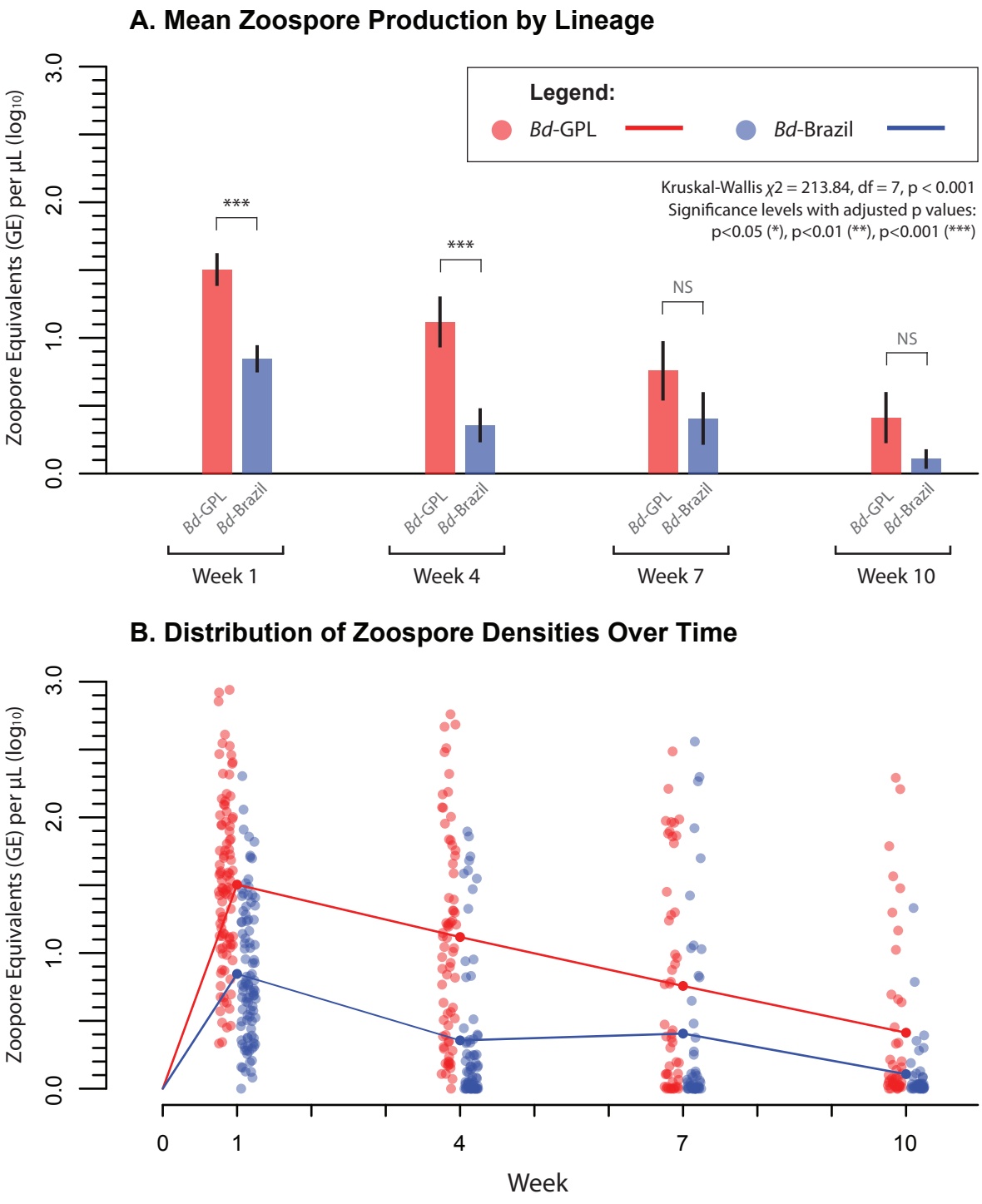
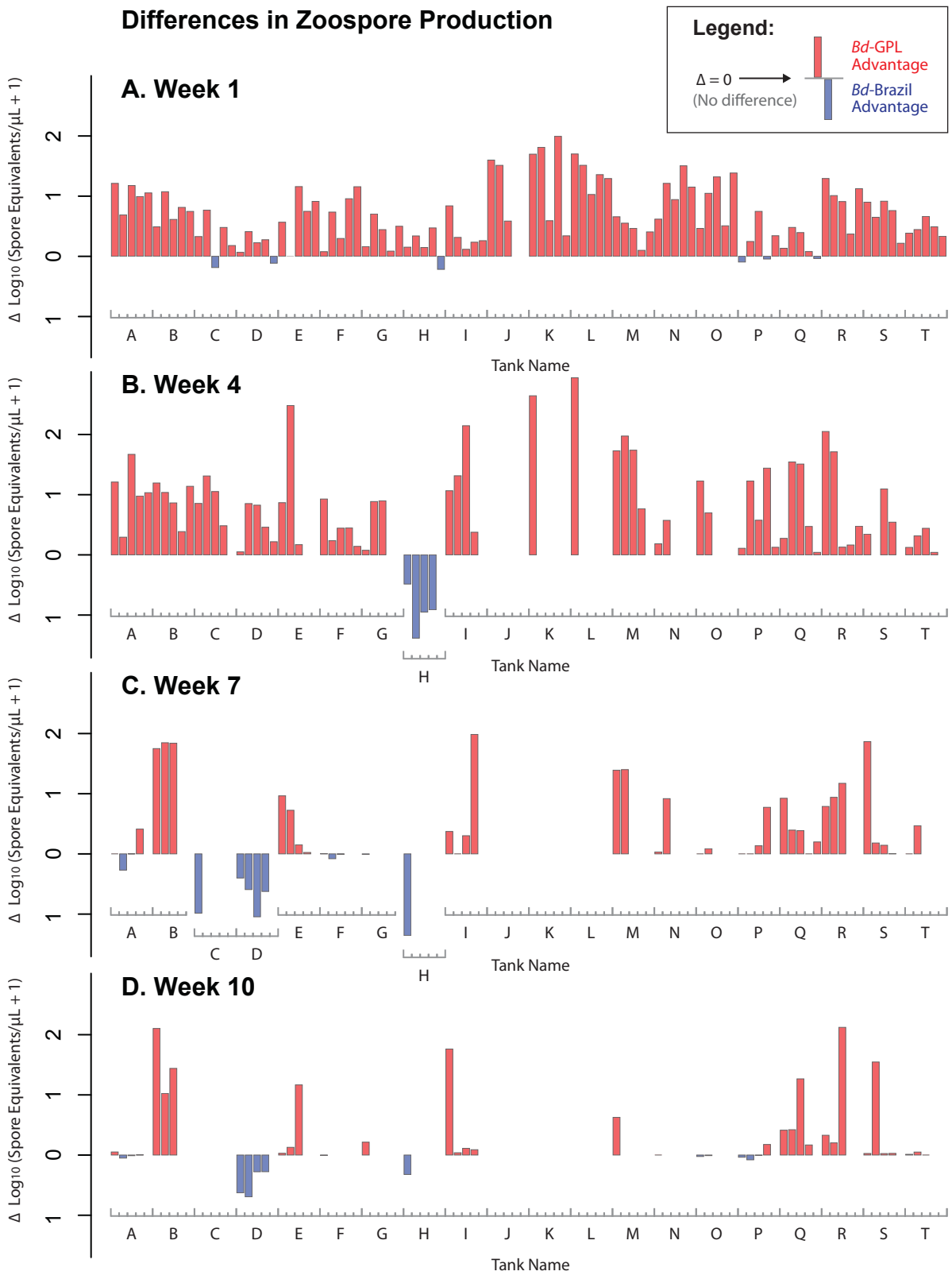


Figure 4-3: Zoospore production by *Bd*-GPL is more robust than *Bd*-Brazil. (A) *Bd*-GPL produces significantly more spores in the early stages of the 10 week coinfection experiment. Bar plots show mean zoospore densities (log (GE/µL + 1)) for *Bd*-GPL (red) and *Bd*-Brazil (blue) at sampled time points. Black bars show the range for 95% confidence intervals. (B) Distributions of individual *Bd*-GPL and *Bd*-Brazil zoospore densities. Data points are offset from

the x-line for visualization. Lines show trends in mean zoospore densities (log (GE/μL + 1)) for each lineage over 10 weeks.

Figure 4-4 (**Next Page**): Differences between paired *Bd*-GPL and *Bd*-GPL zoospore densities; week 1 (A), week 4 (B), week 7 (C), and week 10 (D). Bar plots represent the difference (Δ) between log-transformed *Bd*-GPL and *Bd*-Brazil zoospore densities [Δ = (log (*Bd*-GPL(GE/μL) + 1) - log (*Bd*-Brazil(GE/μL) + 1))] for each individual *H. curtipes* host. Bars with positive values (red) are individuals in which spore densities of *Bd*-GPL are greater than *Bd*-Brazil. Bars with negative values (blue) are individuals in which spore densities of *Bd*-Brazil are greater than *Bd*-GPL. Individuals' tank identifications are shown along the x-axis. Individual data is not tracked from week to week, only within tanks. Missing bars indicate individuals that have either died or cleared the disease over the course of this experiment.


Differences in Zoospore Production
Legend:
Δ = 0
(No difference)
Bd-GPL Advantage
Bd-Brazil Advantage
A. Week 1
B. Week 4
C. Week 7
D. Week 10
Δ Log10 (Spore Equivalents/μL + 1)
Tank Name
A B C D E F G H I J K L M N O P Q R S T

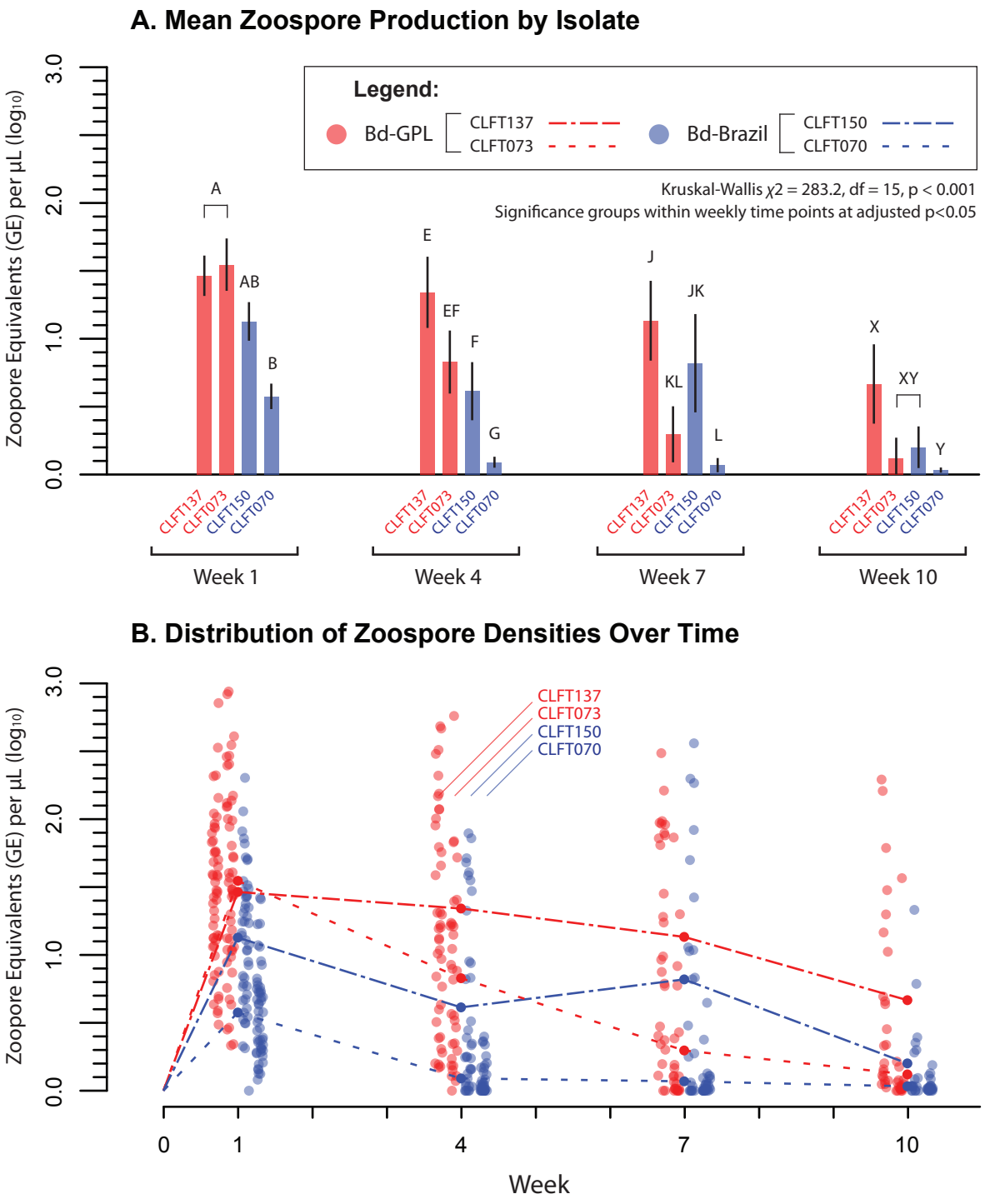


Figure 4-5: Zoospore production varies significantly by isolate strain. (A) Differences spore production during the 10-week coinfection experiment. Bar plots show mean zoospore densities (log (GE/µL + 1)) at sampled time points for the two *Bd*-GPL (red) and the two *Bd*-Brazil (blue) stains studied. Black bars show the range for 95% confidence intervals. (B) Distributions of individual *Bd* zoospore densities partitioned by strain. Data points are offset from the x-line for

visualization (left to right: CLFT173, CLFT073, CLFT150, CLFT070). Lines show the trend in mean zoospore densities (log (GE/µL + 1)) for each strain over 10 weeks.

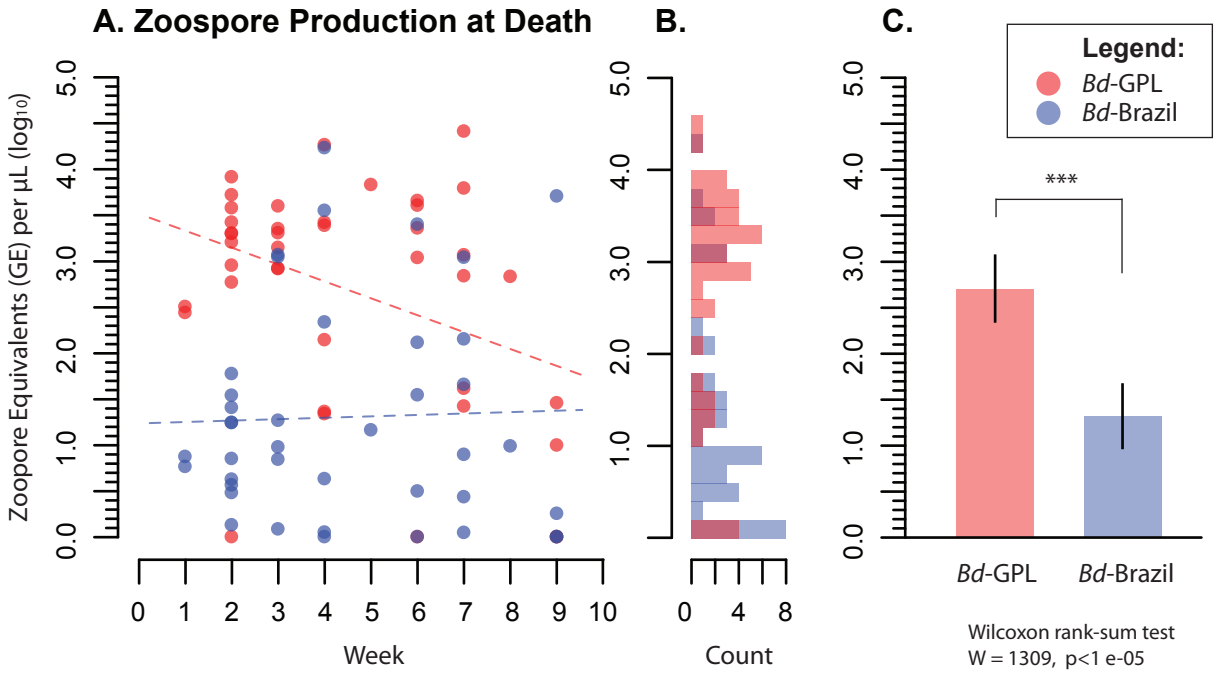


Figure 4-6: Zoospore densities in post-mortem skin swabs. (A) Spore densities (log (GE/µL + 1)) over time. Red dashed line shows the fitted linear model for *Bd*-GPL spore density over 10 weeks (slope = -0.184, $R^2 = 0.140$, p = 0.016), and blue dashed line shows no relationship between *Bd*-Brazil spore density over 10 weeks (slope = 0.014, $R^2 = 0.001$, p = 0.854). (B) Histogram of *Bd*-GPL (red) and *Bd*-Brazil (blue) zoospore density distributions at death. (C) Difference in mean *Bd*-GPL and *Bd*-Brazil zoospore densities at death.

Tables:

Table 4-1: *Bd* isolates used for this experiment

Isolate	Lineage	Year	Passages	Host	Locality
CLFT073	*Bd*-GPL	2013	9	*Aplastodiscus sp.*	Serra dos Orgaos National Park, Rio de Janeiro
CLFT070	*Bd*-Brazil	2013	9	*Hylodes japi*	Serra do Japi, Jundiai, São Paulo
CLFT137	*Bd*-GPL	2014	6	*Hylodes cardosoi*	Serra da Graciosa, Morretes, Parana
CLFT150	*Bd*-Brazil	2014	6	*Hylodes cardosoi*	Serra da Graciosa, Morretes, Parana
CLFT035 *qPCR standard	*Bd*-GPL	2013	9	*Hypsiboas faber*	Reserva Betary, Iporanga São Paulo

Table 4-2: Replicated treatment design for coinfections between *Bd* strains

Tank	Treatment (1-4)	(*Bd*-GPL x *Bd*-Brazil) Inoculum		n Starting	n Surviving
A	1	CLFT073 x CLFT070		5	5
B	2	CLFT137 x CLFT070		5	3
C	4	CLFT137 x CLFT150		5	0
D	4	CLFT137 x CLFT150		5	4
E	2	CLFT137 x CLFT070		5	5
F	1	CLFT073 x CLFT070		5	5
G	3	CLFT073 x CLFT150		5	4
H	4	CLFT137 x CLFT150		5	1
I	2	CLFT137 x CLFT070		5	5
J	3	CLFT073 x CLFT150		5	0
K	1	CLFT073 x CLFT070		5	0
L	2	CLFT137 x CLFT070		5	0
M	4	CLFT137 x CLFT150		5	3
N	3	CLFT073 x CLFT150		5	2
O	1	CLFT073 x CLFT070		5	2
P	3	CLFT073 x CLFT150		5	5
Q	4	CLFT137 x CLFT150		5	5
R	2	CLFT137 x CLFT070		5	3
S	1	CLFT073 x CLFT070		5	5
T	3	CLFT073 x CLFT150		5	5
20 tanks		5 replicates per treatment	Total n:	n = 100	n = 62

Chapter 5

Conclusions and future directions

Our understanding of *Batrachochytrium* biology is growing at a rapid pace. The study system we have developed in the southern Atlantic Forest of Brazil, examining the divergence, hybridization, and competition between *Bd* strains has resulted in significant contributions to the field of amphibian chytrid research. This dissertation described the ranges and ecological histories of newly discovered, divergent *Bd* strains in the Brazilian Atlantic Forest; showed patterns of genome variation in the major lineages and hybrids; and demonstrated that *Bd* strains compete on coinfected host frogs.

***The current day range of a novel, Brazilian lineage of Bd*:**

When novel, divergent strains were first reported from the Atlantic Forest region of southern Brazil (Schloegel et al. 2012), only four samples of *Bd*-Brazil had been observed (three isolated from the wild in São Paulo State, and one isolate from a captive, market bullfrog purchased in Ypsilanti, Michigan; Schloegel et al. 2012, Rosenblum et al. 2013). Additional, questions remained about whether the hybrid strain collected in Paraná State represented a single anomalous event, or if there is ongoing sexual reproduction in this region. In the largest regional sampling of *Bd* published to date (Morgan et al. 2007; Farrer et al. 2013; Rosenblum et al. 2013), this dissertation describes 19 new *Bd*-Brazil isolates, and four new hybrid isolates for genetic and phenotypic studies. We also isolated and genotyped over 70 *Bd*-GPL isolates, giving a much

clearer understanding of *Bd* genotype distributions in the Atlantic Forest. This refined view of genotype distributions over a 2400 Km transect through Atlantic Brazil has allowed us to make key hypotheses and predictions about the nature of lineage contact. Based on our population genetic analyses we put forth the hypothesis that the endemic *Bd*-Brazil is being competitively excluded from its former range in the Atlantic Forest by an invasive, and hypercompetitive *Bd*-GPL (Jenkinson et al. 2016).

***Genome variation in divergent Bd lineages, and in hybrid Bd strains*:**

To understand the causes and consequences of secondary contact we observed in Brazil, we selected 51 of our previously collected field isolates for whole genome sequencing. We showed the degree of genome divergence between *Bd*-GPL and *Bd*-Brazil. In this study, we generated genome sequences for three hybrid strains. With genomic data we reinforced our earlier hypothesis that hybridization was ongoing in the Brazilian Atlantic Forest. We also showed for the first time that hybrid *Bd* strains are able to form viable, backcrossed F2 offspring with parental genotypes in this region. The discovery of the Brazilian hybrid zone is significant because *Bd* was once thought to be a strictly asexual pathogen and, the only known cases of hybridization in this species are restricted to the Atlantic coast of Brazil. Natural hybrids present a unique opportunity to understand the genetic basis of lineage divergence, reproductive incompatibility and the first steps toward speciation in fungal pathogens.

We found that differences exist in unequal hybrid inheritance. We also found that these putative incompatibility loci occurred in clustered blocks throughout the hybrid genomes forming divergence islands. Gene ontology analyses showed that these divergent loci were

enriched for genes responding to oxidative stress, suggesting a potential mechanism underlying the ecological diversification between *Bd* lineages.

***Bd strains compete for transmission in coinfected model hosts*:**

We show for the first time that *Bd* strains compete for resources in laboratory experiments. Competitive differences are significant between *Bd*-GPL and *Bd*-Brazil strains, and most often *Bd*-GPL strains are the superior competitors. We developed and experimental system to test competitive differences between *Bd* lineages, *in-vivo* using a model amphibian host. These results provide experimental support for our phylogeographic hypothesis of a recent/ongoing *Bd*-GPL takeover in the endemic range of *Bd*-Brazil. Finally, we describe a number of new tools and methodological advances for understanding *Bd* evolution. We show the utility of digital PCR as a powerful tool to detect rare population variants in a mixed sample of *Bd* genotypes. We also demonstrate the *Hymenochirus curtipes* system as a scalable model to standardize *Bd* infection studies across laboratories.

The results of this dissertation, and the body of *Bd* research accumulated in the last six years, point toward a number of immediate next steps. First, field sampling pathogen strains should be a research priority. We need both fine-scale sampling in regions with genetic diversity – such as the Brazilian hybrid zone – and broad-scale surveys in understudied regions for *Bd* (continental Asia or the Amazon basin for example). Second, we need to continue refining the toolkit for chytrid research. We need to pursue new tools like digital PCR and newly developed microfluidic, multiplex PCR systems (Byrne et al. 2017) to generate population informative data from smaller quantities of sample DNA without the need to culture isolates, or the need to

sacrifice wild animals. Third, we need more data on phenotypic variation among *Bd* strains, data like virulence, competitive ability, or relative growth rates. A deeper understanding of phenotypic variation in *Bd* is necessary if we are to connect the underlying genetic variation the research community has built to the phenotypes that play key roles in the ecology of *Bd*. Finally, we need to more widely distribute our conclusions and assessments about pathogen transmission risks, and how the ecology of amphibians and amphibian chytrid may shift with the human movement of pathogens. In an example of positive policy changes arising from engaged science communication, the Unites States just banned the importation of live salamanders in 2016 to prevent the introduction of the newly discovered *Batrachochytrium salamandrivorans*. Continued efforts like this are necessary if we wish for our research to inform the control of new hypervirulent disease.

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Appendix A

Supplementary Material for Chapter 2

Tables:

Table A-1: Atlantic Forest Bd isolates analyzed the transect study; with associated collection dates, geographic origins, host species, and collectors

Isolate	Lineage	Year	Geographic Origin, Municipality	State	Host Species	Collector
CLFT021	*Bd*-GPL	2010	Serra do Japi, Cabreúva	SP	Unidentified sp.	L. F. Toledo & C. A. Vieira
CLFT024/02	Hybrid	2011	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	L. F. Toledo & C. A. Vieira
CLFT026	*Bd*-GPL	2011	Reserva Betary, Iporanga	SP	*Hypsiboas faber*	C. Lambertini
CLFT029	*Bd*-GPL	2011	Serra do Japi, Jundiaí	SP	*Hypsiboas albopunctatus*	C. Lambertini
CLFT030	*Bd*-GPL	2012	Bertioga	SP	*Hylodes phyllodes*	C. Lambertini
CLFT031	*Bd*-GPL	2012	Bertioga	SP	*Hylodes phyllodes*	C. Lambertini
CLFT032	*Bd*-GPL	2012	Bertioga	SP	*Hylodes phyllodes*	C. Lambertini
CLFT033	*Bd*-GPL	2012	Bertioga	SP	*Hylodes phyllodes*	C. Lambertini
CLFT034	*Bd*-GPL	2013	Bertioga	SP	*Hylodes phyllodes*	T. S. Jenkinson
CLFT035	*Bd*-GPL	2013	Reserva Betary, Iporanga	SP	*Hypsiboas faber*	K. R. Zamudio
CLFT036	*Bd*-GPL	2013	Reserva Betary, Iporanga	SP	*Hypsiboas faber*	D. Rodriguez
CLFT037	*Bd*-GPL	2013	Reserva Betary, Iporanga	SP	*Hypsiboas faber*	K. R. Zamudio
CLFT038	Hybrid	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	T. S. Jenkinson
CLFT039	Hybrid	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	T. S. Jenkinson
CLFT040	*Bd*-Brazil	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	L. F. Toledo
CLFT041	*Bd*-Brazil	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	D. Rodriguez
CLFT042	*Bd*-GPL	2013	Reserva Betary, Iporanga	SP	*Hypsiboas faber*	C. M. Betancourt
CLFT043	*Bd*-GPL	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	T. S. Jenkinson
CLFT044	*Bd*-Brazil	2013	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	C. M. Betancourt
CLFT045	*Bd*-GPL	2013	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT046	*Bd*-GPL	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	C. M. Betancourt
CLFT047	*Bd*-GPL	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	C. M. Betancourt
CLFT048	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT049	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	T. S. Jenkinson
CLFT050	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT051	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	T. S. Jenkinson
CLFT052	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT053	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	K. R. Zamudio
CLFT054	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	D. Rodriguez
CLFT055	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	T. Y. James
CLFT056	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	T. S. Jenkinson
CLFT057	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT058	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	T. S. Jenkinson
CLFT060	*Bd*-GPL	2013	Pomerode	SC	*Hylodes meridionalis*	T. S. Jenkinson
CLFT061	*Bd*-Brazil	2013	Pomerode	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT062	*Bd*-GPL	2013	Pomerode	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT063	*Bd*-GPL	2013	Pomerode	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT064	*Bd*-GPL	2013	Pomerode	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT065	*Bd*-Brazil	2013	Serra do Japi, Jundiaí	SP	*Hylodes japi*	C. M. Betancourt
CLFT066	*Bd*-Brazil	2013	Serra do Japi, Jundiaí	SP	*Hylodes japi*	J. E. Longcore
CLFT067	*Bd*-Brazil	2013	Serra do Japi, Jundiaí	SP	*Hylodes japi*	C. M. Betancourt
CLFT068	*Bd*-Brazil	2013	Serra do Japi, Jundiaí	SP	*Hylodes japi*	C. M. Betancourt
CLFT070	*Bd*-Brazil	2013	Serra do Japi, Jundiaí	SP	*Hylodes japi*	J. E. Longcore
CLFT071	*Bd*-Brazil	2013	Serra do Japi, Jundiaí	SP	*Hylodes japi*	C. M. Betancourt

CLFT073	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Aplastodiscus* sp.	C. M. Betancourt
CLFT074	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	Unidentified sp.	C. M. Betancourt
CLFT075	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	Unidentified sp.	T. Y. James
CLFT076	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	C. M. Betancourt
CLFT077	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	C. M. Betancourt
CLFT078	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	T. Y. James
CLFT079	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	T. Y. James
CLFT080	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	C. M. Betancourt
CLFT081	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	Unidentified sp.	C. M. Betancourt
CLFT082	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	C. M. Betancourt
CLFT083	*Bd*-GPL	2013	Lago Iacy, Teresópolis	RJ	*Scinax hayii*	C. M. Betancourt
CLFT084	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	C. M. Betancourt
CLFT085	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	Unidentified sp.	C. M. Betancourt
CLFT086	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	Unidentified sp.	C. M. Betancourt
CLFT087	*Bd*-GPL	2013	Lago Iacy, Teresópolis	RJ	*Scinax hayii*	C. M. Betancourt
CLFT088	*Bd*-GPL	2013	Lago Iacy, Teresópolis	RJ	*Scinax hayii*	C. M. Betancourt & T. S. Jenkinson
CLFT095	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Aplastodiscus* sp.	T. S. Jenkinson
CLFT096	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Aplastodiscus* sp.	C. Lambertini
CLFT097	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Aplastodiscus* sp.	A. V. Aguilar
CLFT098	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Aplastodiscus* sp.	C. Lambertini
CLFT099	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Aplastodiscus* sp.	T. S. Jenkinson
CLFT100	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	C. Lambertini
CLFT101	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Aplastodiscus* sp.	A. V. Aguilar & T. S. Jenkinson
CLFT102	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT103	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	A. V. Aguilar
CLFT104	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	A. V. Aguilar
CLFT105	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT106	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT107	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	C. Lambertini
CLFT108	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT109	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT110	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	A. V. Aguilar
CLFT111	*Bd*-GPL	2014	Santa Teresa	ES	*Aplastodiscus* sp.	T. S. Jenkinson
CLFT113	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT114	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT115	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT116	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT117	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	C. Lambertini
CLFT118	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT119	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT120	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT121	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT122	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT123	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	C. Lambertini
CLFT124	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT126	*Bd*-GPL	2014	Vargem Alta	ES	*Phyllomedusa* sp.	A. V. Aguilar
CLFT127	*Bd*-GPL	2014	Vargem Alta	ES	*Dendropsophus minutus*	T. Y. James
CLFT128	*Bd*-GPL	2014	Vargem Alta	ES	*Aplastodiscus* sp.	T. Y. James
CLFT129	*Bd*-GPL	2014	Vargem Alta	ES	*Aplastodiscus* sp.	A. V. Aguilar
CLFT130	*Bd*-GPL	2014	Vargem Alta	ES	*Scinax fuscovarius*	A. V. Aguilar
CLFT131	*Bd*-GPL	2014	Vargem Alta	ES	*Lithobates catesbeianus*	T. S. Jenkinson
CLFT132	*Bd*-GPL	2014	Vargem Alta	ES	*Dendropsophus minutus*	A. V. Aguilar
CLFT133	*Bd*-GPL	2014	Vargem Alta	ES	*Phyllomedusa* sp.	A. V. Aguilar
CLFT134	*Bd*-GPL	2014	Vargem Alta	ES	*Phyllomedusa* sp.	T. S. Jenkinson
CLFT135	*Bd*-GPL	2014	Vargem Alta	ES	*Scinax fuscovarius*	K. R. Zamudio
CLFT136	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	T. S. Jenkinson
CLFT137	*Bd*-GPL	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT138	*Bd*-GPL	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	C. Lambertini
CLFT139	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT141	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	L. F. Moreno de Lima
CLFT142	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Crossodactylus schmidti*	P. P. Morão
CLFT143	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT144	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT145	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	P. P. Morão
CLFT146	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT148	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson

CLFT149	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT150	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	P. P. Morão
CLFT151	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	P. P. Morão
CLFT152	*Bd*-GPL	2014	Serra da Graciosa, Morretes	PR	*Crossodactylus schmidti*	T. S. Jenkinson
CLFT153	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
JEL648	*Bd*-Brazil	2010	Serra do Japi, Jundiaí	SP	*Hylodes japi*	J. E. Longcore
JEL649	*Bd*-Brazil	2010	Serra do Japi, Jundiaí	SP	*Hylodes japi*	J. E. Longcore
LMS902	*Bd*-GPL	2008	Pindamonhangaba (farm)	SP	*Lithobates catesbeianus*	L. M. Schloegel
LMS925	*Bd*-GPL	2008	Pindamonhangaba (farm)	SP	*Lithobates catesbeianus*	L. M. Schloegel
LMS929	*Bd*-GPL	2008	Belém (farm)	PA	*Lithobates catesbeianus*	L. M. Schloegel
LMS931	*Bd*-GPL	2009	Tremembé (farm)	SP	*Lithobates catesbeianus*	L. M. Schloegel
UM142	*Bd*-Brazil	2009	Ypsilanti, U.S.A. (market)	MI	*Lithobates catesbeianus*	T. Y. James

Brazilian state abbreviations are: Bahia (BA), Espírito Santo (ES), Rio de Janeiro (RJ), São Paulo (SP), Paraná (PR), and Santa Catarina (SC).

Table A-2: Locus specific F_{IS} values of by population and lineage

Population	Locus											
	8009 X2	BdC5	BdSC 2.0	BdSC 3.1	BdSC 4.16	R6046	BdSC 6.8	BdSC 6.15	BdSC 7.6	BdSC 9.1	BdSC 11.5	BdSC 16.2
***Bd*-GPL**												
Serra Bonita, BA	-1.000	-1.000	-0.714		-0.090		-1.000		-1.000	-0.090	-0.500	
Santa Teresa, ES	-0.090	-0.500	-0.363	-0.200			-0.363		-0.714		-1.000	
Vargem Alta, ES	-0.500	-1.000	-0.363	-0.200	-0.090				-0.714	-0.363	-0.714	
Serra dos Órgãos & Teresópolis, RJ	-0.368	-0.733	-0.695	0.216	-0.314		-0.397		-0.130	-0.857	-0.314	
Serra do Japi, SP		-1.000	-1.000	1.000	-1.000					-1.000	-1.000	
Bertioga, SP		-1.000	0.272		-0.600		-0.090		-0.090	-0.333	-0.600	
Reserva Betary, SP		-0.200	1.000	-0.200	-0.200	1.000	-1.000	-0.200	-0.200	-1.000	-1.000	
Serra da Graciosa, PR	-1.000	-1.000	0.142	0.625	-0.363	1.000	0.400		-0.200	-1.000	-0.363	
Pomerode, SC		-0.333	-0.333		1.000		-0.333		-1.000	-0.333	-1.000	
Rancho Queimado, SC	-0.297	-0.777	-0.230		0.058		-0.777		-0.600	-0.066	-1.000	
***Bd*-Brazil**												
Serra do Japi, SP			-0.666	-0.428		-0.111	-0.250	-0.250		-1.000		-0.250
Serra da Graciosa, PR				-0.363		0.142	-1.000	-1.000		-0.531		-1.000

Blank values indicate monomorphic markers within a population, shaded values correspond to significant deviation from Hardy-Weinberg equilibrium in per-locus exact tests (significance $\alpha = 0.05$).

Appendix B

Supplementary Material for Chapter 3

Figures:

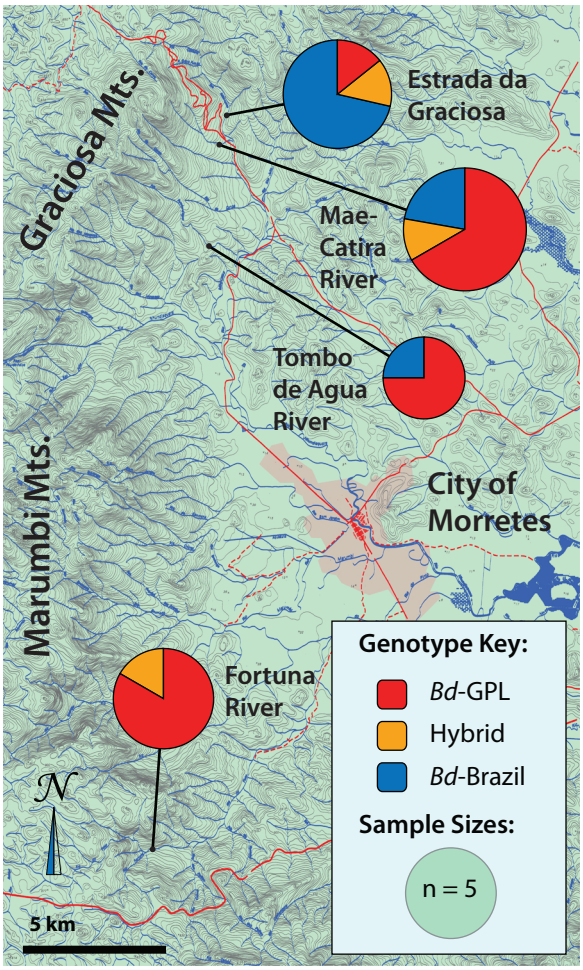


Figure B-1: Detail map of the Serra da Graciosa hybrid zone. Distributions of *Bd*-GPL (red), *Bd*-Brazil (blue), and hybrid strains (yellow) are shown.

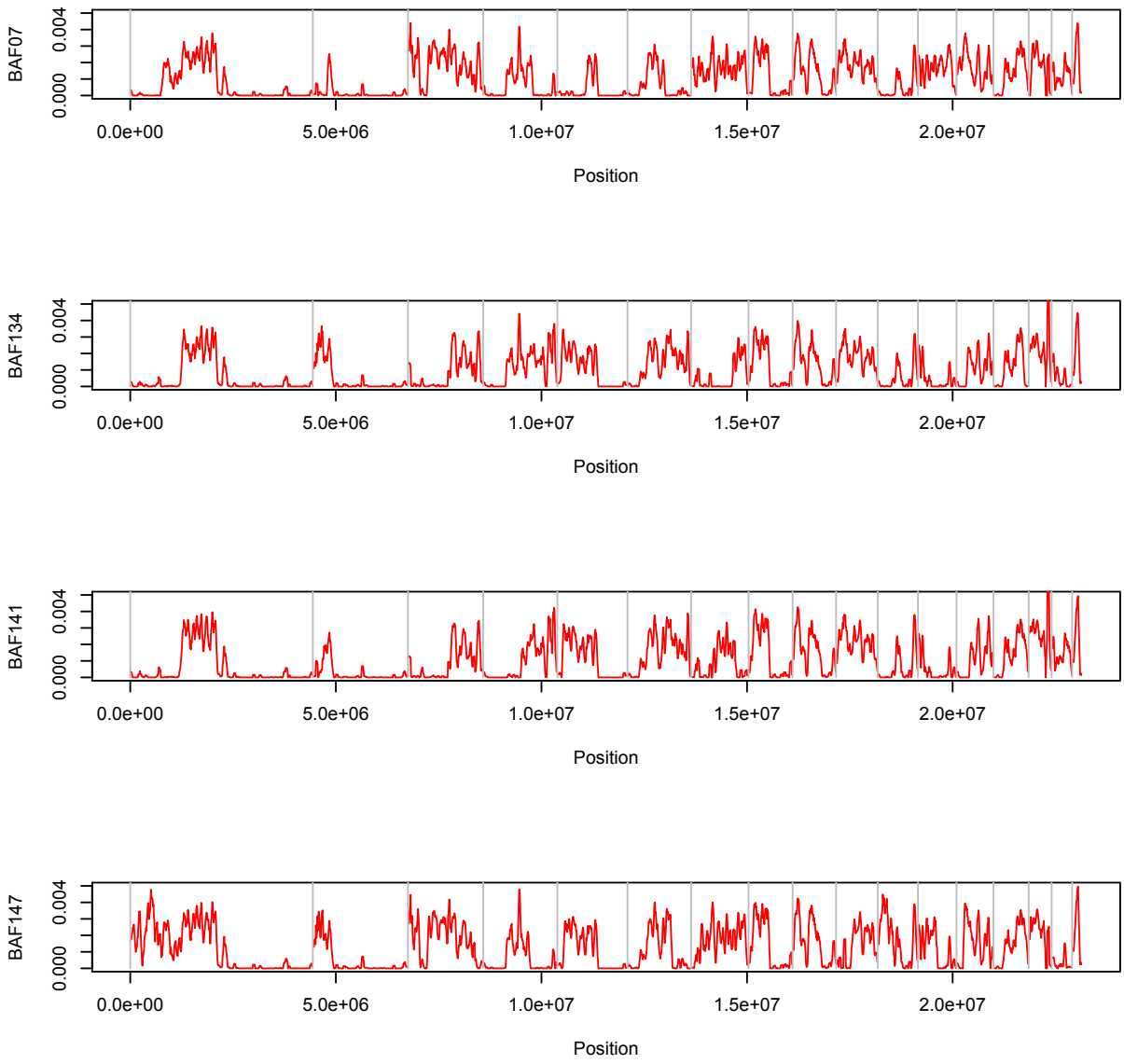
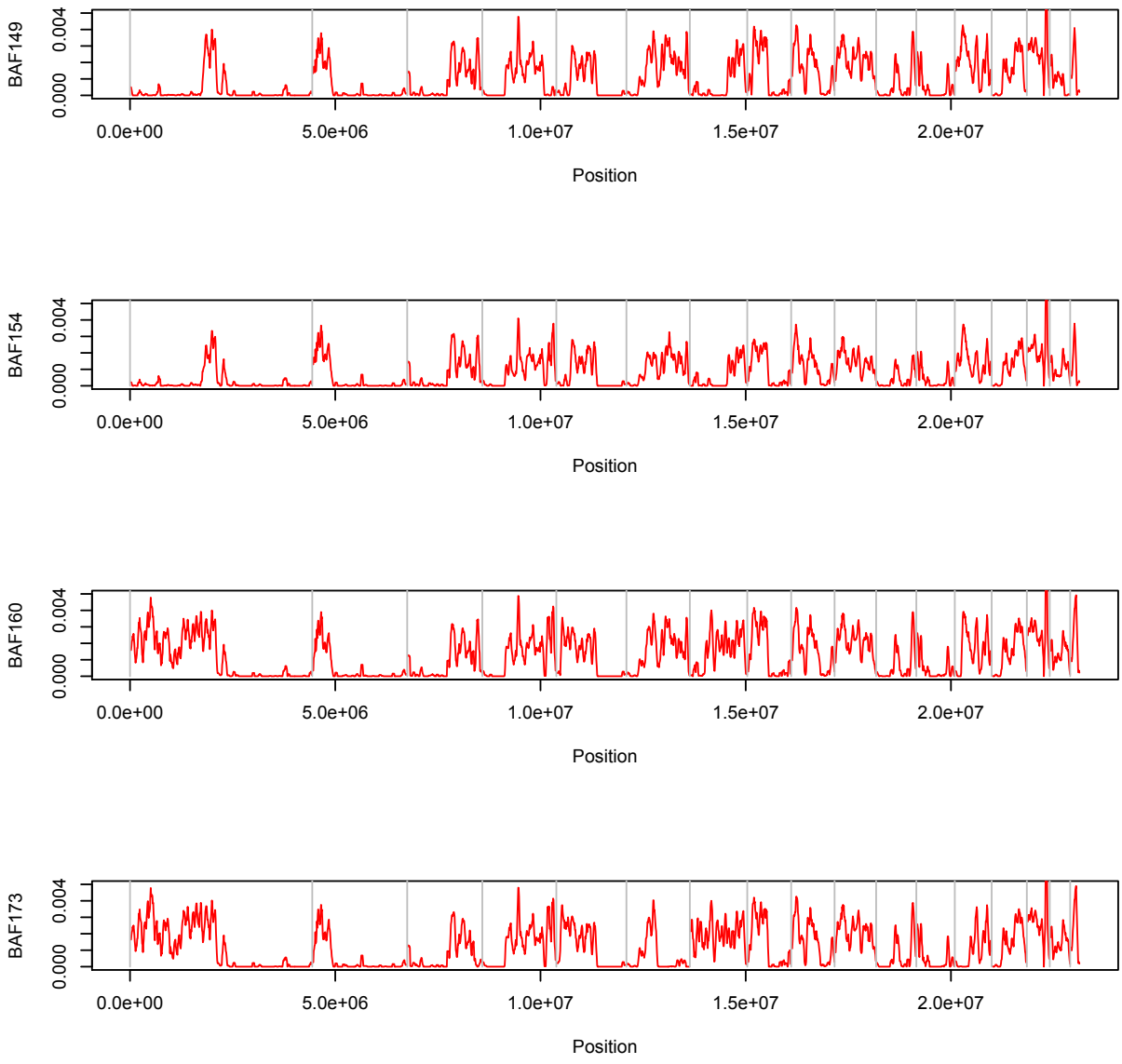
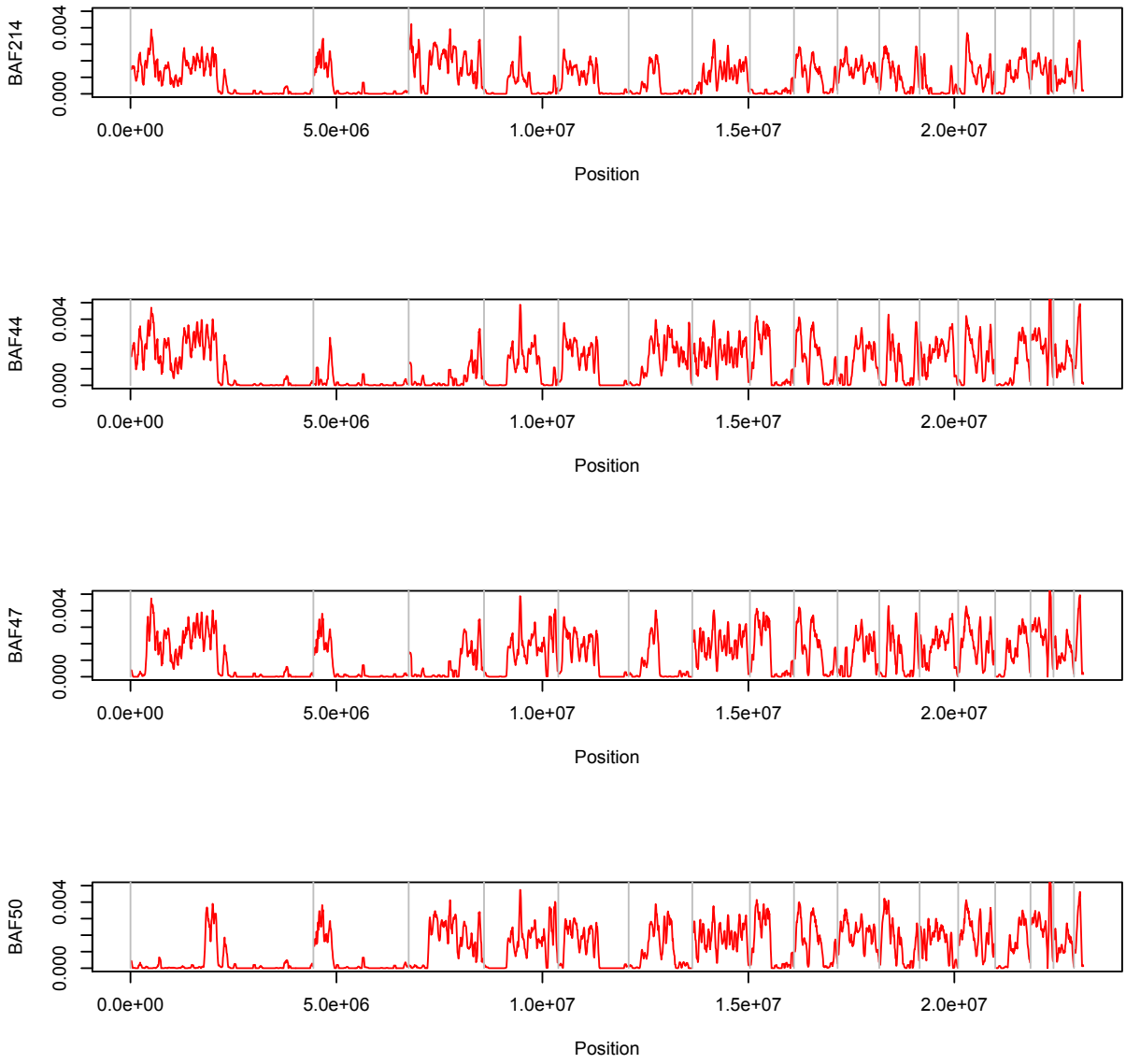
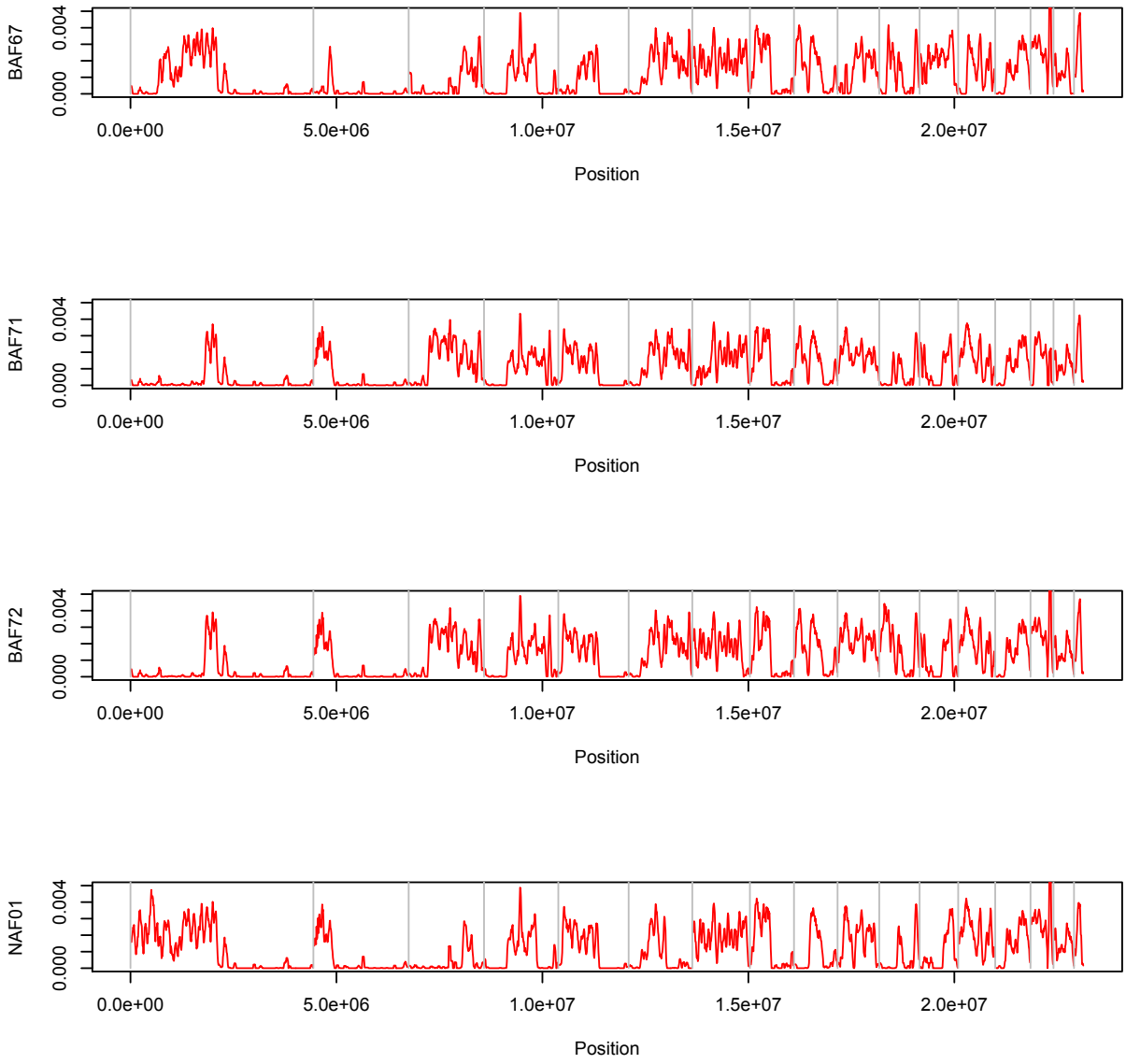


Figure B-2 (**This and the Next 12 Pages**): Genome-wide heterozygosity plots across 50kb sliding windows. % heterozygosity (y- axis, H_O) plots across a 50kb sliding windows. The plots on pages 129 to 137 are heterozygosity plots for *Bd*-GPL isolates, plots from pages 138 - 140 are for *Bd*-Brazil isolates, and the plots on page 141 are for hybrid isolates. The plots are marked with synonymous field codes for the isolation names. Starting positions of chromosomes are shown in gray lines.


BAF149
0.000
0.004
0.0e+00
5.0e+06
1.0e+07
1.5e+07
2.0e+07
Position
BAF154
0.000
0.004
0.0e+00
5.0e+06
1.0e+07
1.5e+07
2.0e+07
Position
BAF160
0.000
0.004
0.0e+00
5.0e+06
1.0e+07
1.5e+07
2.0e+07
Position
BAF173
0.000
0.004
0.0e+00
5.0e+06
1.0e+07
1.5e+07
2.0e+07
Position


BAF214
0.000
0.004
0.0e+00
5.0e+06
1.0e+07
1.5e+07
2.0e+07
Position
BAF44
0.000
0.004
0.0e+00
5.0e+06
1.0e+07
1.5e+07
2.0e+07
Position
BAF47
0.000
0.004
0.0e+00
5.0e+06
1.0e+07
1.5e+07
2.0e+07
Position
BAF50
0.000
0.004
0.0e+00
5.0e+06
1.0e+07
1.5e+07
2.0e+07
Position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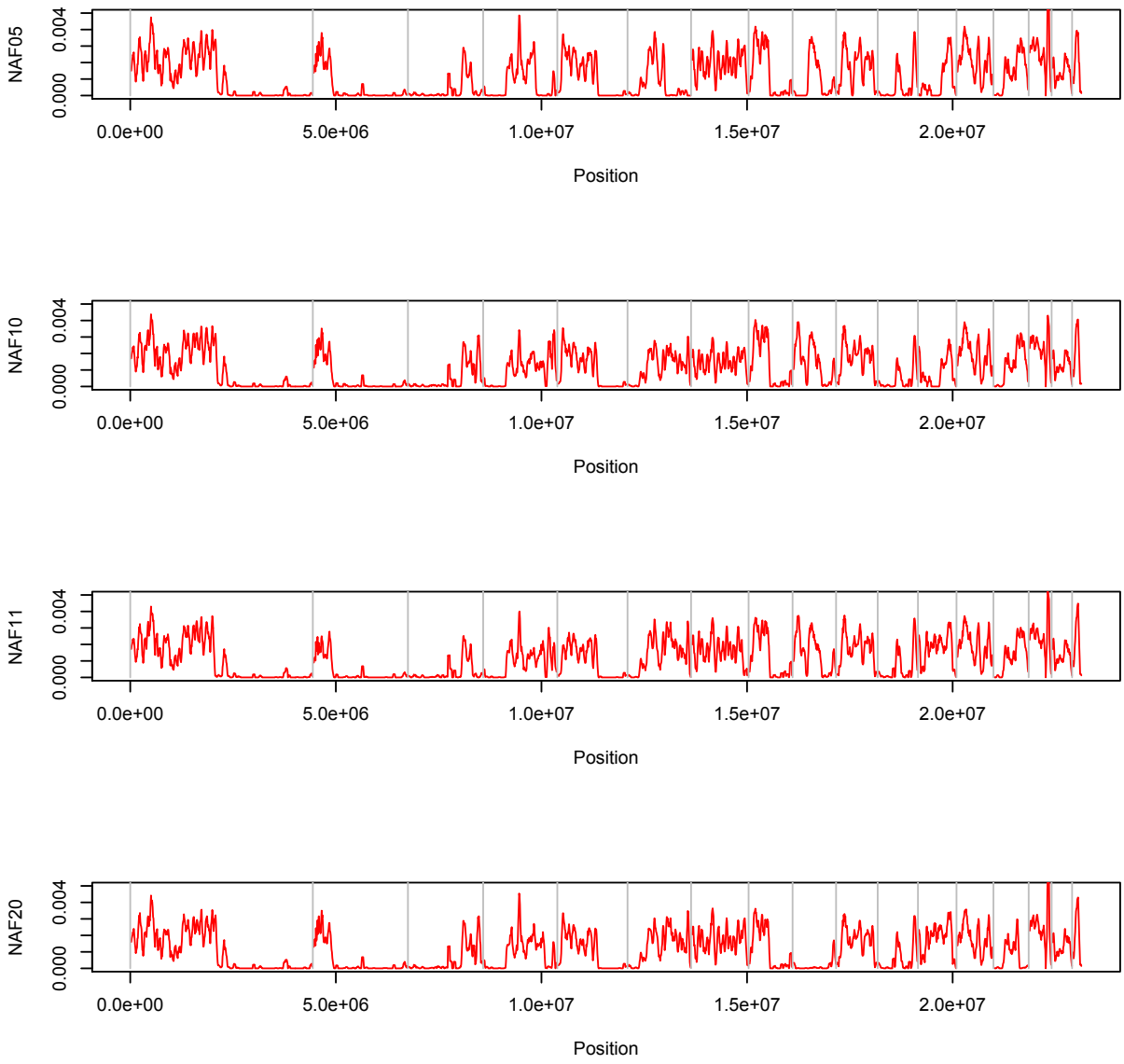
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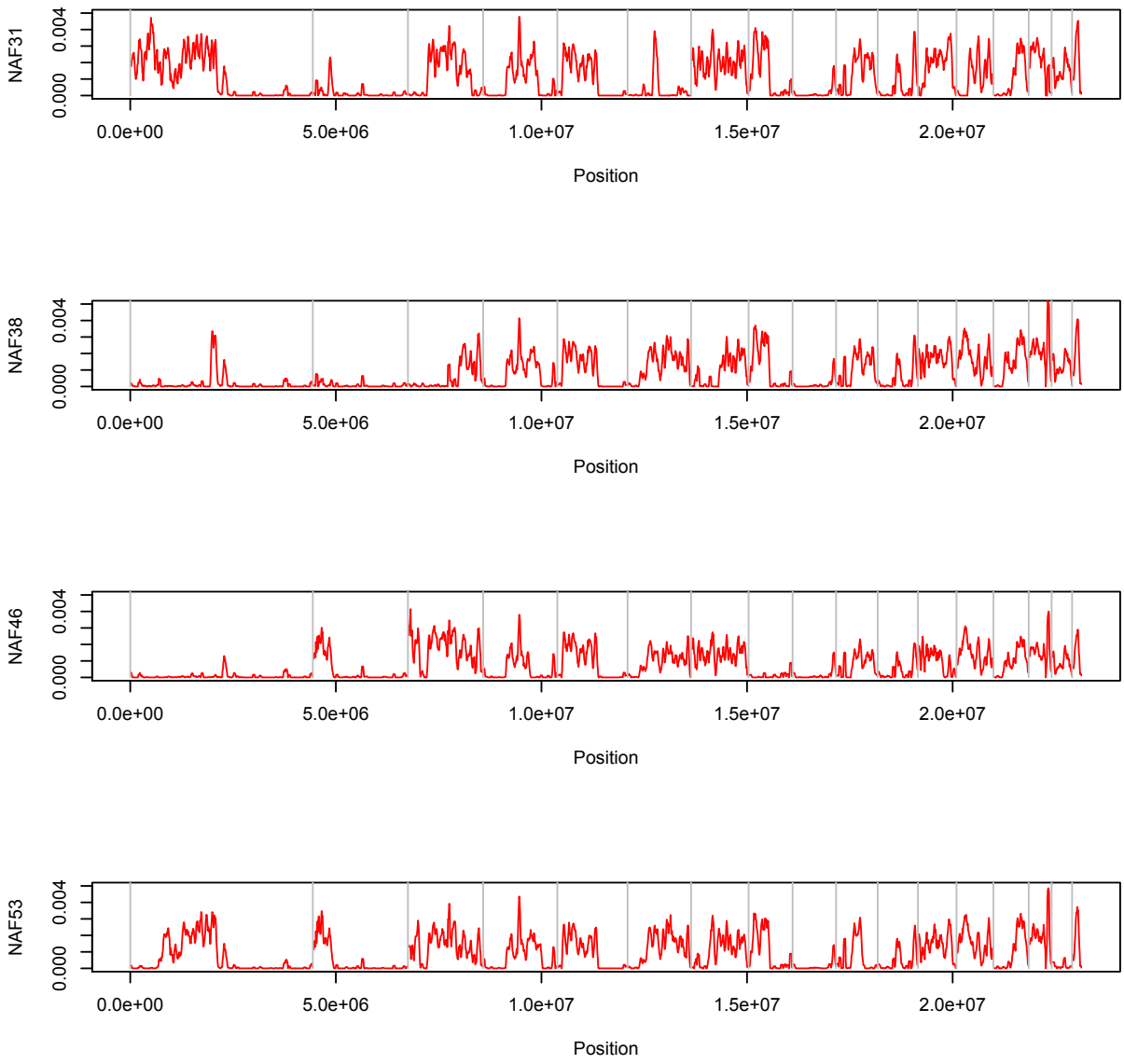
Position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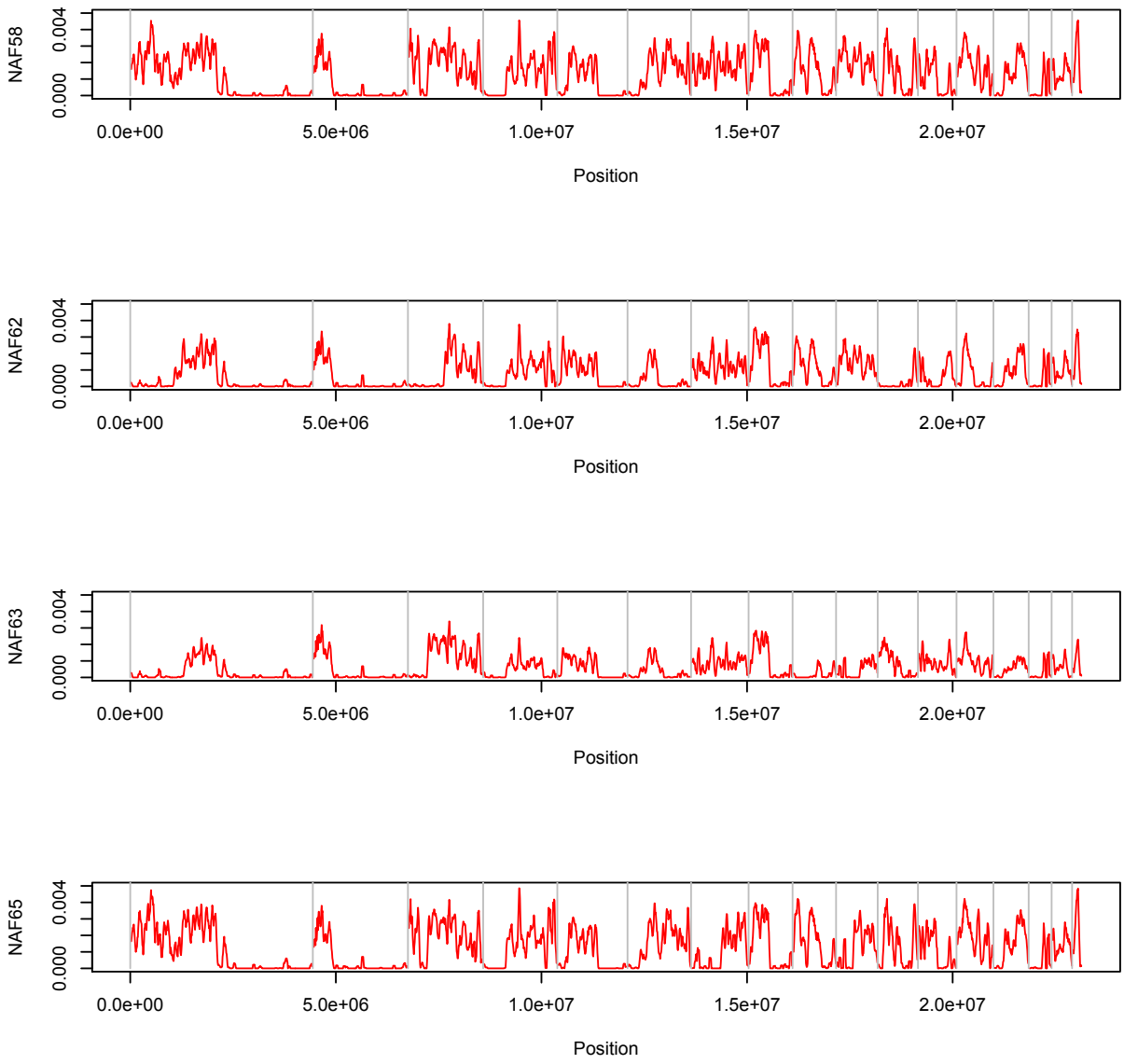
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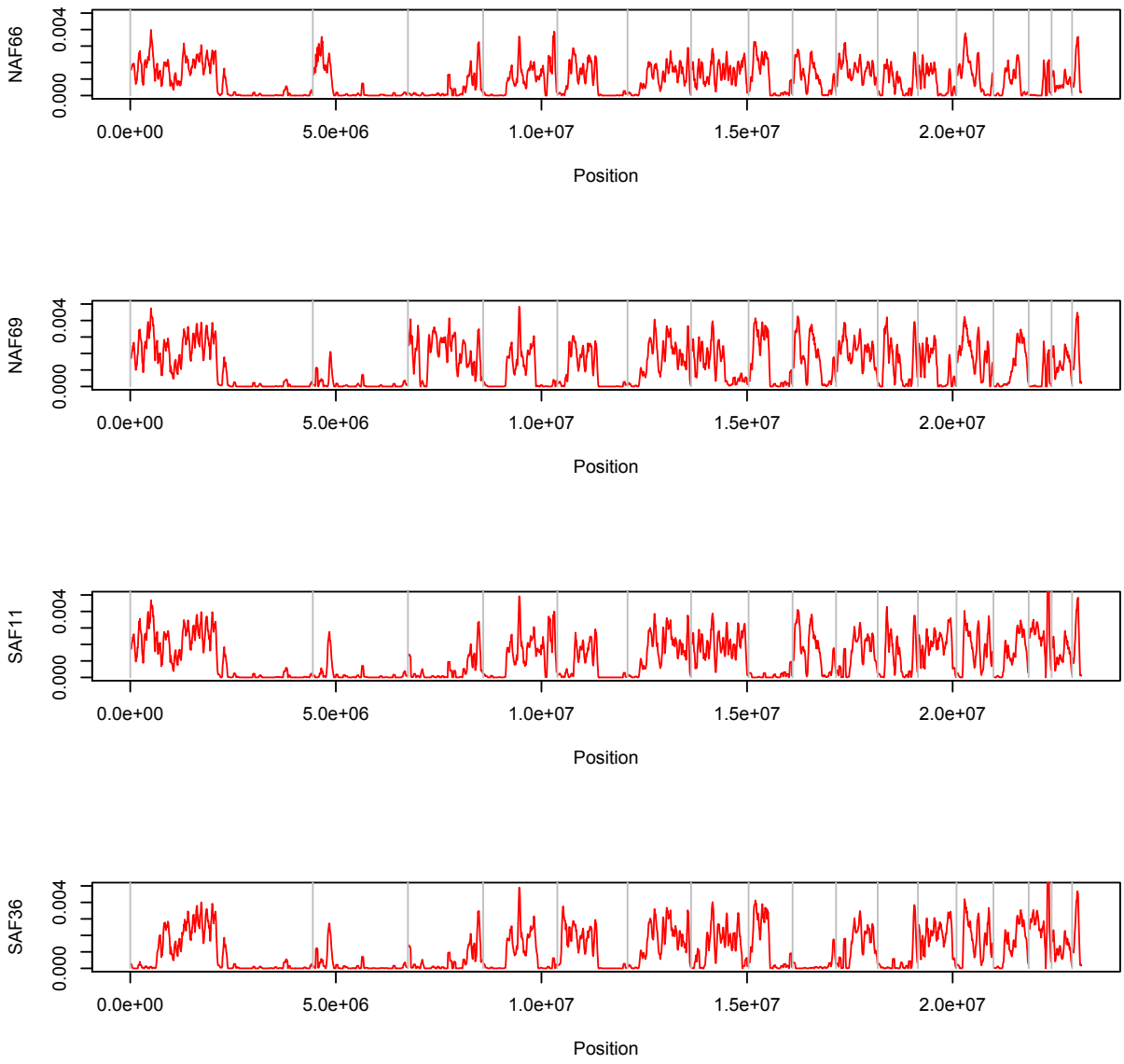
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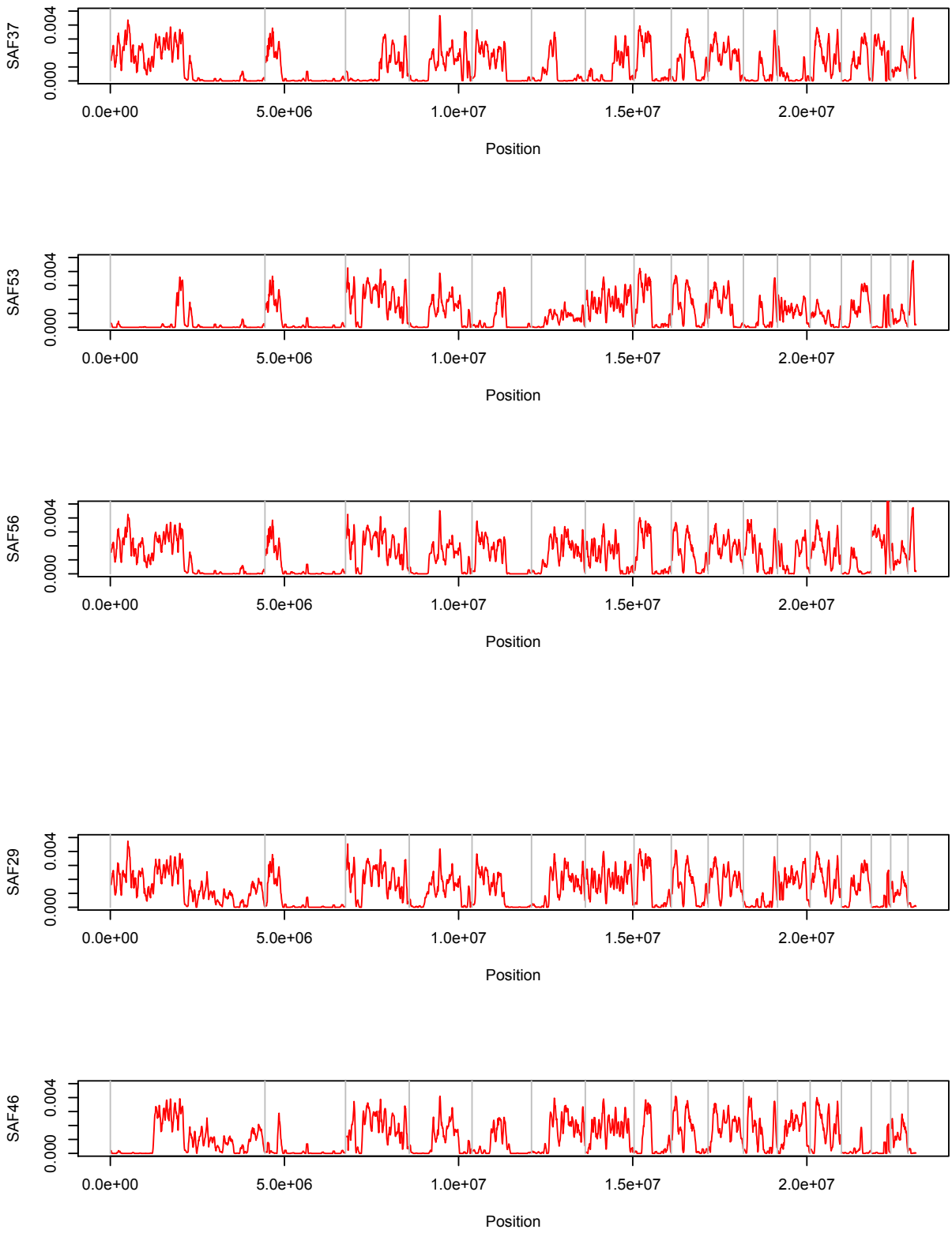
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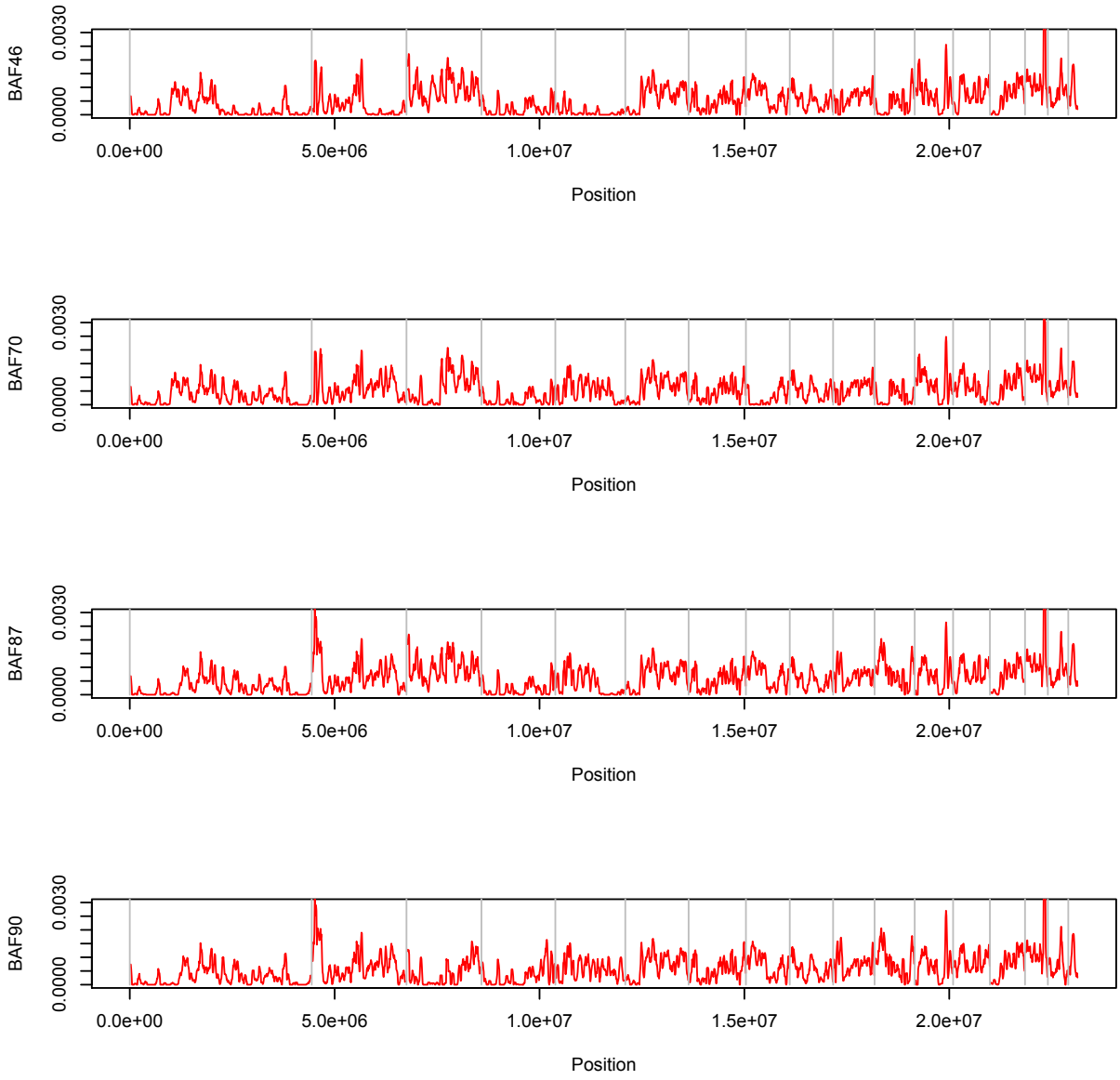
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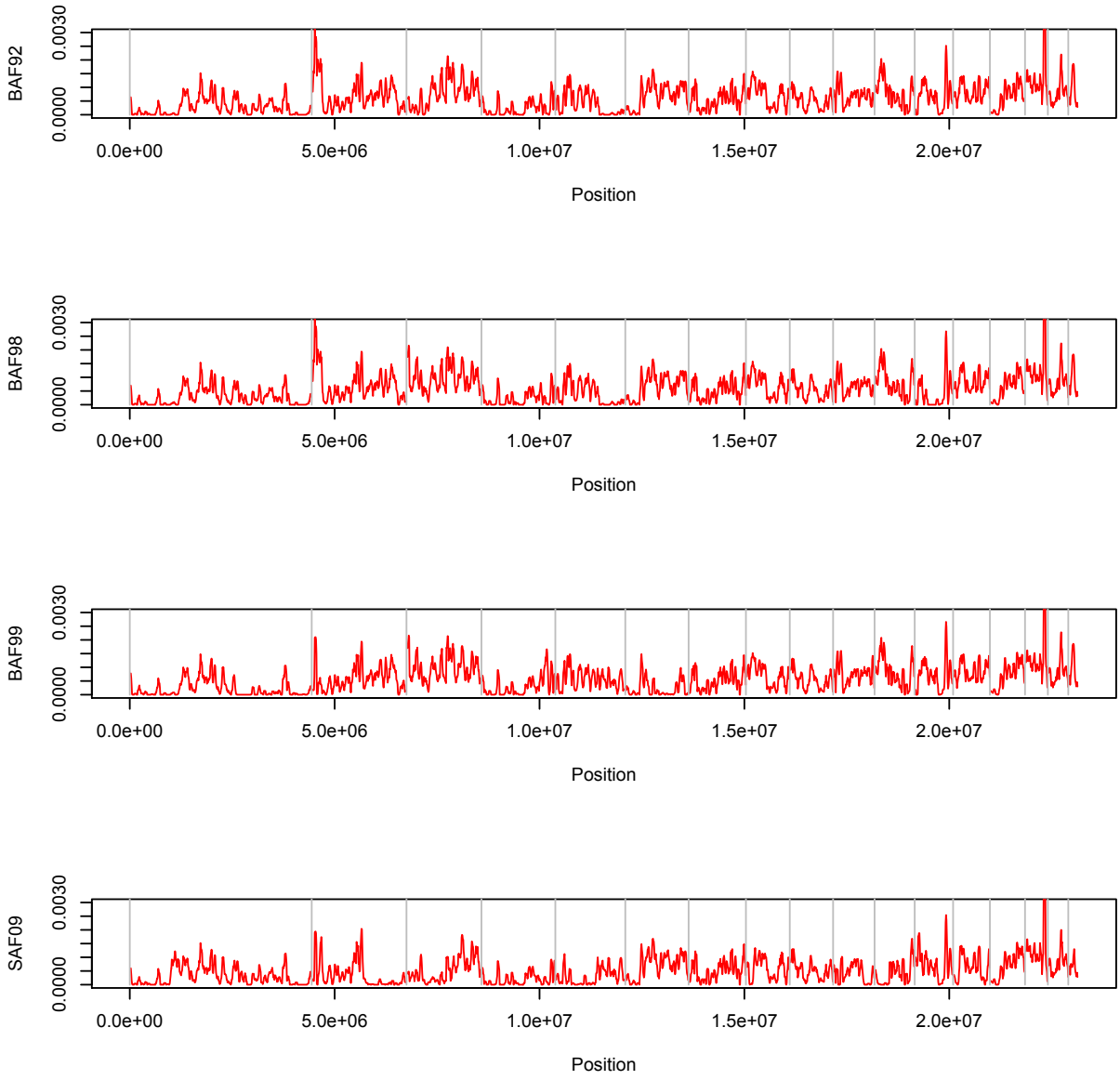
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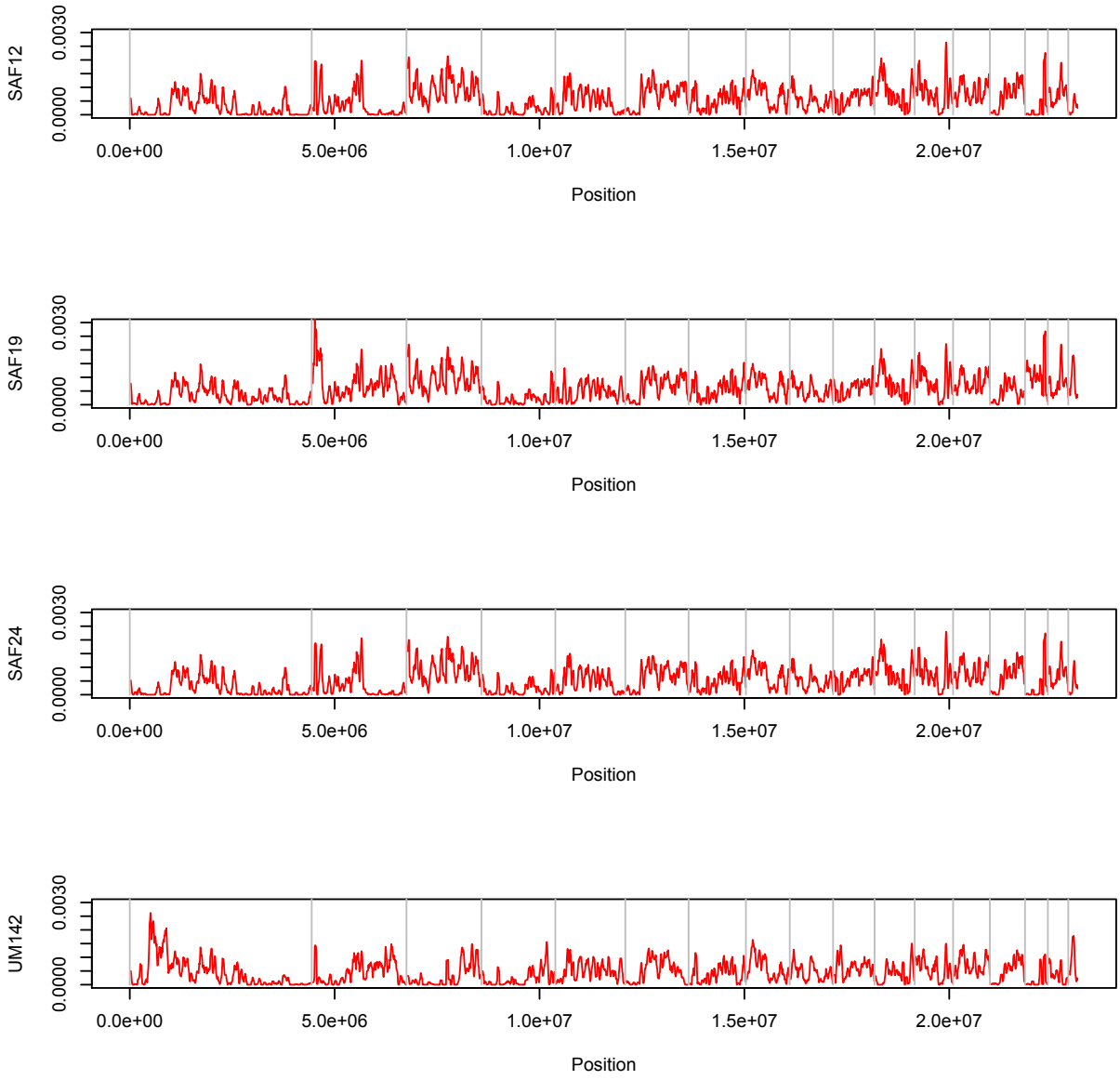
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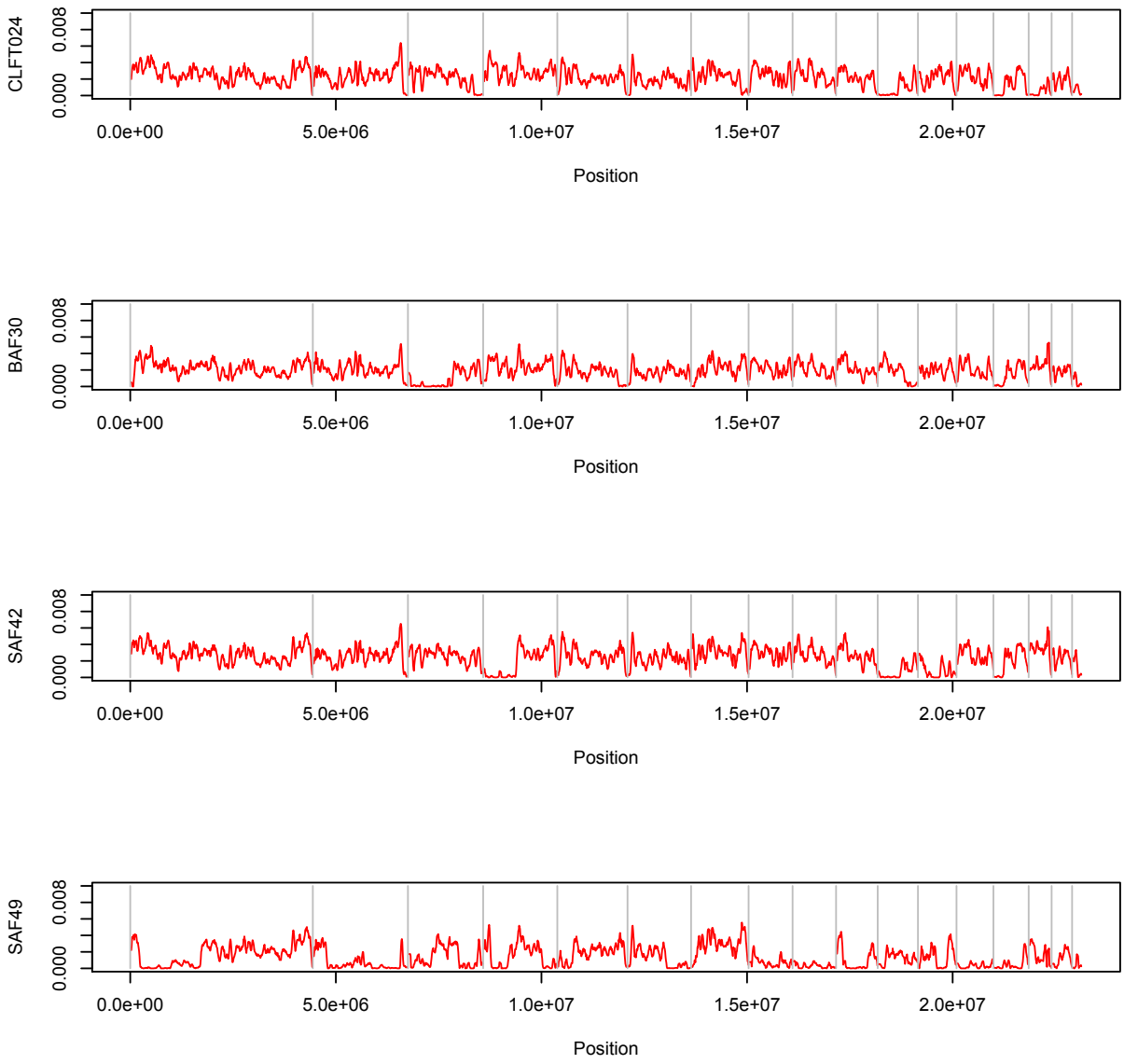
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Table B-1: Previously published *Bd* genomes used in our meta-analysis. All sequences were downloaded from NCBI SRA.

NCBI SSR	Isolate	Reference:
SRR630154	BLI1	Farrer *et al*. (2013)
SRR630155	JEL423	Farrer *et al*. (2013)
SRR630411	ETH4	Farrer *et al*. (2013)
SRR630414	ACON	Farrer *et al*. (2013)
SRR630421	VC1	Farrer *et al*. (2013)
SRR630423	MODS27	Farrer *et al*. (2013)
SRR630564	SP10	Farrer *et al*. (2013)
SRR630566	RC5.1	Farrer *et al*. (2013)
SRR630571	MAD	Farrer *et al*. (2013)
SRR630586	MG4	Farrer *et al*. (2013)
SRR630592	MODS28	Farrer *et al*. (2013)
SRR630604	TF5a1	Farrer *et al*. (2013)
SRR630607	SA1d	Farrer *et al*. (2013)
SRR630610	ETH2	Farrer *et al*. (2013)
SRR630615	AUL	Farrer *et al*. (2013)
SRR630616	MG1	Farrer *et al*. (2013)
SRR633640	BEW2	Farrer *et al*. (2013)
SRR633648	AP15	Farrer *et al*. (2013)
SRR633660	CON2A	Farrer *et al*. (2013)
SRR634028	SA4c	Farrer *et al*. (2013)
SRR634029	SFBC014	Farrer *et al*. (2013)
SRR632144	CJB4	Rosenblum *et al.* (2013)
SRR634692	CJB5-2	Rosenblum *et al.* (2013)
SRR634693	CJB7	Rosenblum *et al.* (2013)
SRR634780	CLFT021	Rosenblum *et al.* (2013)
SRR634821	CLFT023	Rosenblum *et al.* (2013)
SRR634844	CLFT024	Rosenblum *et al.* (2013)
SRR634964	CLFT024-02	Rosenblum *et al.* (2013)
SRR634966	CLFT026	Rosenblum *et al.* (2013)
SRR634967	EV001	Rosenblum *et al.* (2013)
SRR634975	JEL238	Rosenblum *et al.* (2013)
SRR634976	JEL267	Rosenblum *et al.* (2013)
SRR634977	JEL271	Rosenblum *et al.* (2013)
SRR634978	JEL275	Rosenblum *et al.* (2013)
SRR634979	JEL289	Rosenblum *et al.* (2013)
SRR634981	JEL310	Rosenblum *et al.* (2013)
SRR634983	JEL359	Rosenblum *et al.* (2013)
SRR635020	JEL408	Rosenblum *et al.* (2013)
SRR635068	JEL427	Rosenblum *et al.* (2013)
SRR635198	JEL433	Rosenblum *et al.* (2013)

SRR635199	JEL627	Rosenblum *et al.* (2013)
SRR635200	LBAbercrom	Rosenblum *et al.* (2013)
SRR635201	JEL429	Rosenblum *et al.* (2013)
SRR635202	LFT001	Rosenblum *et al.* (2013)
SRR635204	MexMkt	Rosenblum *et al.* (2013)
SRR635205	MLA1	Rosenblum *et al.* (2013)
SRR635206	NBRC106979	Rosenblum *et al.* (2013)
SRR635207	SRS812	Rosenblum *et al.* (2013)
SRR635208	TST75	Rosenblum *et al.* (2013)
SRR635210	UM142	Rosenblum *et al.* (2013)

Table B-2: Results of the GO functional enrichment analysis

Rank	GO MFID	P value	Function
1	GO:0020037	2.94E-06	heme binding
2	GO:0046906	2.94E-06	tetrapyrrole binding
3	GO:0046872	0.000200217	metal ion binding
4	GO:0043169	0.000241327	cation binding
5	GO:0004784	0.000593667	superoxide dismutase activity
6	GO:0016721	0.000593667	oxidoreductase activity, acting on superoxide radicals as acceptor
7	GO:0000287	0.000932158	magnesium ion binding
8	GO:0016874	0.004526303	ligase activity
9	GO:0016491	0.005174977	oxidoreductase activity
10	GO:0030246	0.005376076	carbohydrate binding
11	GO:0016879	0.005910811	ligase activity, forming carbon-nitrogen bonds
12	GO:0005506	0.006489343	iron ion binding
13	GO:0008603	0.007714765	cAMP-dependent protein kinase regulator activity
14	GO:0004550	0.007714765	nucleoside diphosphate kinase activity
15	GO:0010181	0.007999703	FMN binding
16	GO:0016209	0.013009595	antioxidant activity
17	GO:0030234	0.013459823	enzyme regulator activity
18	GO:0000155	0.016603325	phosphorelay sensor kinase activity
19	GO:0004733	0.016603325	pyridoxamine-phosphate oxidase activity
20	GO:0016641	0.016603325	oxidoreductase activity, acting on the CH-NH2 group of donors, oxygen as acceptor
21	GO:0016775	0.016603325	phosphotransferase activity, nitrogenous group as acceptor
22	GO:0004673	0.016603325	protein histidine kinase activity
23	GO:0016885	0.016603325	ligase activity, forming carbon-carbon bonds
24	GO:0046914	0.021271492	transition metal ion binding
25	GO:0016881	0.024489315	acid-amino acid ligase activity
26	GO:0016620	0.025845412	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor

27	GO:0019887	0.031576615	protein kinase regulator activity
28	GO:0016638	0.031576615	oxidoreductase activity, acting on the CH-NH2 group of donors
29	GO:0003684	0.031576615	damaged DNA binding
30	GO:0019207	0.031576615	kinase regulator activity
31	GO:0043167	0.032002526	ion binding
32	GO:0098772	0.038274991	molecular function regulator
33	GO:0002161	0.045541832	aminoacyl-tRNA editing activity
34	GO:0004749	0.045541832	ribose phosphate diphosphokinase activity
35	GO:0008897	0.045541832	holo-[acyl-carrier-protein] synthase activity
36	GO:0016705	0.045652763	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen

Appendix C

Supplementary Material for Chapter 4

Figures:

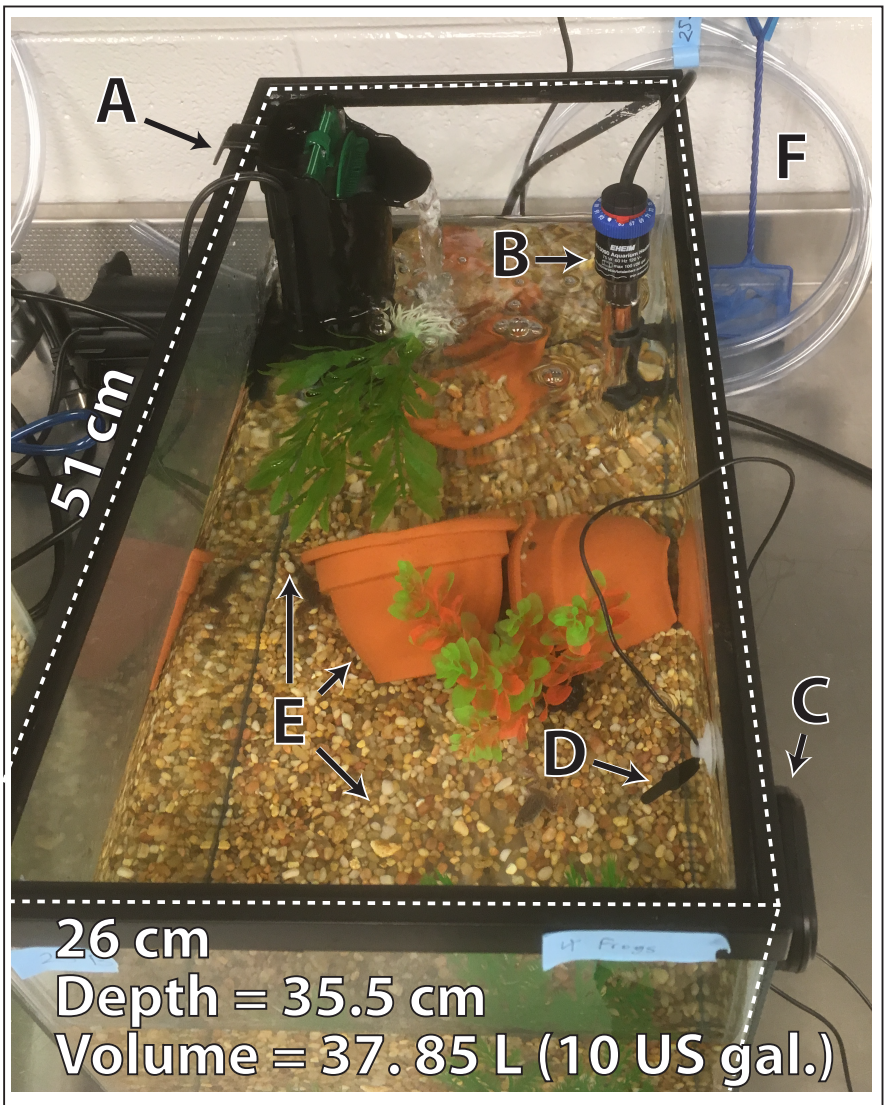

Figure C-1: Set-up and dimensions of the experimental tanks. Components include: filter (A), aquarium heater (B), external digital thermometer (C) with probe (D). Environmental enrichment included gravel substrate, aquarium plants and broken terra cotta shelters.

Tables:

Table C-1: Nucleotide primers, probes and reaction conditions for quantitative real-time PCR (qPCR) assays.

Primers and Probes for qPCR		
Description	Sequence	Features
Forward qPCR primer ITS-1chytr	5' - CCTTGATATAATACAGTGTGCCATATGTC - 3'	Published in: (Boyle et al. 2007)
Reverse qPCR primer 5.8s chytr	5' - AGCCAAGAGATCCGTTGTCAAA - 3'	Published in: (Boyle et al. 2007)
Bd specific TaqMan probe (Boyle et al. 2007)	5' -FAM- CGAGTCGAACAAAAT -MGBNFQ- 3'	FAM fluorescence MGB quencher
Reaction Components for qPCR		
	Reagent	Volume / rxn
	TaqMan Fast Advanced qPCR Master Mix (Thermo Fisher Inc.)	12.50 µL
	H_2O	2.75 µL
	Forward Primer (18 µM)	1.25 µL
	Reverse Primer (18 µM)	1.25 µL
	TaqMan probe (5 µM)	1.25 µL
	BSA (400ng/µL)	1.00 µL
	Diluted DNA extract (1:10)	5.00 µL
	Total	**25 µL**
Thermocycling Conditions for qPCR		
	Step	Time
	95º initial denature	0:20
	95º denature	0:01
	60º anneal/extension	0:20
	x 50 Cycles	

Table C-2: Nucleotide primers, probes and reaction conditions for digital PCR (dPCR) assays.

Primers and Probes for dPCR		
Description	Sequence	Features
Forward dPCR primer (Rodriguez et al. in prep)	5' - CTACCATCTAATAATTCCACCATCCCATT - 3'	
Reverse dPCR primer (Rodriguez et al. in prep)	5' - CACCCTATCATTCTTTTATAACCTTAGCCATA - 3'	
Bd-GPL strain-specific TaqMan probe (Rodriguez et al. in prep)	5' -VIC- AAGGTAGTACAGGTAAACC -MGBNFQ- 3'	VIC fluorescence MGB quencher
Bd-Brazil strain-specific TaqMan probe (Rodriguez et al. in prep)	5' -FAM- AGGTAGTACAGATAAACC -MGBNFQ- 3'	FAM fluorescence MGB quencher
Reaction Components for dPCR		
	Reagent	Volume / rxn
	QuantStudio 3D dPCR Master Mix v2 (Thermo Fisher Inc.)	7.25 µL
	TaqMan Custom Assay (20x)	0.725 µL
	H_2O	1.525 µL

Template DNA extract (diluted 1:1 - 1:5, optimized by qPCR)	5.00 µL
Total	**14.5 µL**

Thermocycling Conditions for dPCR	
Step	Time
96º initial denature	10:00
60º anneal/extension	2:00
98º denature	3:30
x 50 Cycles	
60º final extension	2:00

Table C-3: Results of post hoc Dunn's multiple comparison test for lineage by time; Kruskal-Wallis comparison of zoospore density (GE/µL) by lineage and time.

	Comparison	Z	P.unadj	P.adj
1	B1 - B10	7.1249327	1.041316e-12	2.915684e-11
2	B1 - B4	5.5535408	2.799403e-08	7.838328e-07
3	B10 - B4	-2.2655558	2.347859e-02	6.574006e-01
4	B1 - B7	5.2330438	1.667412e-07	4.668753e-06
5	B10 - B7	-1.8554048	6.353844e-02	1.000000e+00
6	B4 - B7	0.2696713	7.874131e-01	1.000000e+00
7	B1 - G1	-4.7540247	1.994067e-06	5.583387e-05
8	B10 - G1	-10.8377288	2.280627e-27	6.385755e-26
9	B4 - G1	-9.9112902	3.718133e-23	1.041077e-21
10	B7 - G1	-9.1146887	7.889662e-20	2.209105e-18
11	B1 - G10	4.2034333	2.628966e-05	7.361105e-04
12	B10 - G10	-2.4779202	1.321507e-02	3.700220e-01
13	B4 - G10	-0.4999785	6.170902e-01	1.000000e+00
14	B7 - G10	-0.7020410	4.826536e-01	1.000000e+00
15	G1 - G10	7.9162295	2.448216e-15	6.855006e-14
16	B1 - G4	-1.3480618	1.776385e-01	1.000000e+00
17	B10 - G4	-7.8317976	4.809434e-15	1.346642e-13
18	B4 - G4	-6.4086310	1.468321e-10	4.111299e-09
19	B7 - G4	-6.0611262	1.351717e-09	3.784808e-08
20	G1 - G4	3.0096876	2.615165e-03	7.322462e-02
21	G10 - G4	-5.0662632	4.057009e-07	1.135962e-05
22	B1 - G7	1.7897428	7.349527e-02	1.000000e+00
23	B10 - G7	-4.7385130	2.152922e-06	6.028182e-05
24	B4 - G7	-2.9741668	2.937853e-03	8.225988e-02
25	B7 - G7	-2.9819862	2.863849e-03	8.018778e-02
26	G1 - G7	5.6713877	1.416455e-08	3.966073e-07
27	G10 - G7	-2.1810672	2.917845e-02	8.169965e-01
28	G4 - G7	2.8172881	4.843107e-03	1.356070e-01

Table C-4: Results of post hoc Dunn's multiple comparison test for isolate by time; Kruskal-Wallis comparison of zoospore density (GE/µL) by isolate and time.

	Comparison	Z	P.unadj	P.adj
1	10_1 - 10_10	3.92056313	8.834229e-05	1.060107e-02
2	10_1 - 10_4	1.04901137	2.941729e-01	1.000000e+00
3	10_10 - 10_4	-2.92434454	3.451825e-03	4.142189e-01
4	10_1 - 10_7	1.85613996	6.343361e-02	1.000000e+00
5	10_10 - 10_7	-1.91889410	5.499774e-02	1.000000e+00
6	10_4 - 10_7	0.88633833	3.754352e-01	1.000000e+00
7	10_1 - 134_1	-0.14036926	8.883682e-01	1.000000e+00
8	10_10 - 134_1	-4.00692917	6.151330e-05	7.381596e-03
9	10_4 - 134_1	-1.17192286	2.412280e-01	1.000000e+00
10	10_7 - 134_1	-1.96063746	4.992133e-02	1.000000e+00
11	10_1 - 134_10	7.36073712	1.828974e-13	2.194769e-11
12	10_10 - 134_10	3.13887480	1.695979e-03	2.035175e-01
13	10_4 - 134_10	6.29858694	3.003712e-10	3.604455e-08
14	10_7 - 134_10	5.09855147	3.422625e-07	4.107150e-05
15	134_1 - 134_10	7.42388685	1.137323e-13	1.364788e-11
16	10_1 - 134_4	3.30415610	9.526277e-04	1.143153e-01
17	10_10 - 134_4	-0.84465427	3.983039e-01	1.000000e+00
18	10_4 - 134_4	2.22662691	2.597222e-02	1.000000e+00
19	10_7 - 134_4	1.18538213	2.358664e-01	1.000000e+00
20	134_1 - 134_4	3.40122482	6.708463e-04	8.050156e-02
21	134_10 - 134_4	-4.15658799	3.230358e-05	3.876429e-03
22	10_1 - 134_7	6.39111809	1.646771e-10	1.976126e-08
23	10_10 - 134_7	2.17066620	2.995641e-02	1.000000e+00
24	10_4 - 134_7	5.32180081	1.027450e-07	1.232941e-05
25	10_7 - 134_7	4.14958887	3.330730e-05	3.996875e-03
26	134_1 - 134_7	6.46098804	1.040215e-10	1.248259e-08
27	134_10 - 134_7	-1.01101011	3.120116e-01	1.000000e+00
28	134_4 - 134_7	3.15593101	1.599867e-03	1.919840e-01
29	10_1 - 26_1	1.71409988	8.651038e-02	1.000000e+00
30	10_10 - 26_1	-2.52924239	1.143091e-02	1.000000e+00
31	10_4 - 26_1	0.57847311	5.629447e-01	1.000000e+00
32	10_7 - 26_1	-0.40288339	6.870340e-01	1.000000e+00
33	134_1 - 26_1	1.83583078	6.638269e-02	1.000000e+00
34	134_10 - 26_1	-6.01586892	1.789243e-09	2.147092e-07
35	134_4 - 26_1	-1.77487133	7.591914e-02	1.000000e+00
36	134_7 - 26_1	-5.00549556	5.571850e-07	6.686220e-05
37	10_1 - 26_10	6.54141596	6.093908e-11	7.312689e-09
38	10_10 - 26_10	2.50034316	1.240731e-02	1.000000e+00
39	10_4 - 26_10	5.52858468	3.228246e-08	3.873895e-06
40	10_7 - 26_10	4.40690883	1.048563e-05	1.258276e-03
41	134_1 - 26_10	6.60885963	3.872917e-11	4.647501e-09
42	134_10 - 26_10	-0.57603733	5.645900e-01	1.000000e+00
43	134_4 - 26_10	3.45827451	5.436472e-04	6.523766e-02
44	134_7 - 26_10	0.40812219	6.831840e-01	1.000000e+00

45	26_1 - 26_10	5.22628990	1.729451e-07	2.075342e-05
46	10_1 - 26_4	5.06814638	4.017084e-07	4.820501e-05
47	10_10 - 26_4	0.44961810	6.529858e-01	1.000000e+00
48	10_4 - 26_4	3.85346735	1.164568e-04	1.397482e-02
49	10_7 - 26_4	2.61014604	9.050358e-03	1.000000e+00
50	134_1 - 26_4	5.15316789	2.561225e-07	3.073470e-05
51	134_10 - 26_4	-3.01077864	2.605787e-03	3.126945e-01
52	134_4 - 26_4	1.43852766	1.502844e-01	1.000000e+00
53	134_7 - 26_4	-1.94855333	5.134879e-02	1.000000e+00
54	26_1 - 26_4	3.45351885	5.533236e-04	6.639883e-02
55	26_10 - 26_4	-2.31031607	2.087066e-02	1.000000e+00
56	10_1 - 26_7	3.92784618	8.571000e-05	1.028520e-02
57	10_10 - 26_7	0.05741433	9.542151e-01	1.000000e+00
58	10_4 - 26_7	2.94754367	3.203095e-03	3.843714e-01
59	10_7 - 26_7	1.95538265	5.053792e-02	1.000000e+00
60	134_1 - 26_7	4.01324852	5.988880e-05	7.186656e-03
61	134_10 - 26_7	-3.05079305	2.282378e-03	2.738854e-01
62	134_4 - 26_7	0.89526444	3.706457e-01	1.000000e+00
63	134_7 - 26_7	-2.09015553	3.660383e-02	1.000000e+00
64	26_1 - 26_7	2.55775604	1.053500e-02	1.000000e+00
65	26_10 - 26_7	-2.42035615	1.550531e-02	1.000000e+00
66	26_4 - 26_7	-0.38023950	7.037676e-01	1.000000e+00
67	10_1 - 98_1	4.85689508	1.192407e-06	1.430889e-04
68	10_10 - 98_1	-0.06510308	9.480919e-01	1.000000e+00
69	10_4 - 98_1	3.53011323	4.153819e-04	4.984582e-02
70	10_7 - 98_1	2.21119781	2.702214e-02	1.000000e+00
71	134_1 - 98_1	4.94744871	7.519251e-07	9.023101e-05
72	134_10 - 98_1	-3.68926955	2.248988e-04	2.698786e-02
73	134_4 - 98_1	0.94509169	3.446121e-01	1.000000e+00
74	134_7 - 98_1	-2.59430855	9.478138e-03	1.000000e+00
75	26_1 - 98_1	3.09297958	1.981578e-03	2.377893e-01
76	26_10 - 98_1	-2.93707410	3.313249e-03	3.975899e-01
77	26_4 - 98_1	-0.62412905	5.325428e-01	1.000000e+00
78	26_7 - 98_1	-0.13103664	8.957463e-01	1.000000e+00
79	10_1 - 98_10	8.58274325	9.263696e-18	1.111644e-15
80	10_10 - 98_10	3.91900994	8.891345e-05	1.066961e-02
81	10_4 - 98_10	7.39276736	1.438040e-13	1.725648e-11
82	10_7 - 98_10	6.01707812	1.775934e-09	2.131121e-07
83	134_1 - 98_10	8.63879208	5.681046e-18	6.817255e-16
84	134_10 - 98_10	0.60728693	5.436605e-01	1.000000e+00
85	134_4 - 98_10	5.06085989	4.173698e-07	5.008438e-05
86	134_7 - 98_10	1.68118649	9.272670e-02	1.000000e+00
87	26_1 - 98_10	7.13984252	9.343791e-13	1.121255e-10
88	26_10 - 98_10	1.19970456	2.302541e-01	1.000000e+00
89	26_4 - 98_10	3.88402817	1.027400e-04	1.232880e-02
90	26_7 - 98_10	3.81629675	1.354696e-04	1.625635e-02
91	98_1 - 98_10	4.67106180	2.996468e-06	3.595761e-04
92	10_1 - 98_4	8.91969735	4.675642e-19	5.610770e-17

93	10_10	- 98_4	3.64362274	2.688274e-04	3.225928e-02
94	10_4	- 98_4	7.53181590	5.003948e-14	6.004738e-12
95	10_7	- 98_4	5.94403597	2.780885e-09	3.337062e-07
96	134_1	- 98_4	8.97176409	2.918029e-19	3.501634e-17
97	134_10	- 98_4	0.06541981	9.478398e-01	1.000000e+00
98	134_4	- 98_4	4.90793296	9.204130e-07	1.104496e-04
99	134_7	- 98_4	1.21545087	2.241942e-01	1.000000e+00
100	26_1	- 98_4	7.28582212	3.197146e-13	3.836575e-11
101	26_10	- 98_4	0.71194840	4.764967e-01	1.000000e+00
102	26_4	- 98_4	3.61447489	3.009570e-04	3.611484e-02
103	26_7	- 98_4	3.53172627	4.128565e-04	4.954278e-02
104	98_1	- 98_4	4.51213688	6.417775e-06	7.701330e-04
105	98_10	- 98_4	-0.62457455	5.322503e-01	1.000000e+00
106	10_1	- 98_7	8.58426665	9.141771e-18	1.097012e-15
107	10_10	- 98_7	3.74393477	1.811607e-04	2.173928e-02
108	10_4	- 98_7	7.33763652	2.173986e-13	2.608783e-11
109	10_7	- 98_7	5.90390722	3.549919e-09	4.259902e-07
110	134_1	- 98_7	8.64014599	5.614124e-18	6.736948e-16
111	134_10	- 98_7	0.34798718	7.278498e-01	1.000000e+00
112	134_4	- 98_7	4.91871410	8.711460e-07	1.045375e-04
113	134_7	- 98_7	1.44499542	1.484592e-01	1.000000e+00
114	26_1	- 98_7	7.08239191	1.416873e-12	1.700247e-10
115	26_10	- 98_7	0.95911264	3.375020e-01	1.000000e+00
116	26_4	- 98_7	3.70139626	2.144163e-04	2.572996e-02
117	26_7	- 98_7	3.63920165	2.734846e-04	3.281815e-02
118	98_1	- 98_7	4.51692889	6.274296e-06	7.529156e-04
119	98_10	- 98_7	-0.28944681	7.722395e-01	1.000000e+00
120	98_4	- 98_7	0.32921552	7.419928e-01	1.000000e+00

Appendix D

Standard operating procedure (SOP) for vertebrate animal experiments performed under University of Michigan IACUC protocol: PRO00005605 [1]

Project: Evolutionary Consequences of Pathogen Strain Competition in an Emerging Disease

Room: Life Sciences Institute, Rm. 1241

PI: Timothy Y. James

Species: *Hymenochirus curtipes*

Emergency Contacts: Timothy James (Principal Investigator), email: — @umich.edu

Office: ###-###-####

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Thomas Jenkinson (Ph.D. Student), email: — @umich.edu

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Contents:

[1] The University of Michigan Institutional Animal Care and Use Committee (IACUC) approved versions of this Standard Operating Procedure in April 2016 and May 2017.

D.1 Purpose

This SOP describes the care and handling of animals for experimental chytrid fungus inoculations on laboratory populations of *Hymenochirus curtipes* (dwarf clawed frogs).

D.2 Definitions

1. *Hymenochirus curtipes*: The dwarf clawed frog; an aquatic frog native to tropical West Africa.
2. Animal Treatment Report (ATR): Report written by husbandry personnel describing an animal health concern. ATRs are completed and submitted to veterinary personnel.
3. ULAM: the University of Michigan Unit for Animal Laboratory Medicine

D.3 Responsibility

Animal care and daily monitoring for the experiment will be performed by personnel from the laboratory of Dr. Timothy James.

D.4 Procedures

D.4.1. Feeding and Health Checks

A. Animals will receive a daily health check for moribund or unhealthy animals. James Laboratory personnel will be responsible for daily health monitoring including on holidays and weekends.

B. Feed animals frozen brine shrimp or bloodworms every 2-3 days at a rate such that feeding lasts for five minutes per individual.

C. Remove any uneaten food.

D. Perform a visual inspection for signs of lethargy, bloating or changes in body shape and unresponsiveness to stimuli.

E. Submit an ATR for any concerns listed above. Refer to the ULAM Animal Care Identification and Communication Procedures SOP for detals.

F. Moribund or unhealthy animals will be swabbed with a medical dry swab (Medical Wire and Equipment, MW100 or MW 113) on the ventrum near abdomen and by 5 sweeps on the skin between each of the limb digits, euthanized in a solution of pharmaceutical grade MS-222 (500mg-1g/L) buffered with sodium bicarbonate to achieve a pH of 7.0 to 7.5 in order to reduce skin irritation. Personnel will wear PPE including eye protection and gloves when handling MS-222.

D.4.2. Temperature and Recording

A. Record room temperature and water temperature from at least one aquarium on the room sheet.

B. After quarantine period (see 8. Newly Received Frog Procedures below), maintain water and room temperature between 66°F – 70°F.

C. Report water and room temperatures that are + or – 4 degrees above or below the normal range.

D.4.3. Procedures for Handling Frogs

A. Careful handling is critical to avoid injury to frog skin and the mucous layer.

B. Minimize handling of the animals to necessary experimental procedures.

C. Wear moistened latex or nitrile gloves when handling animals.

D. Change gloves between cages.

E. Aquaria, nets, and other husbandry tools will be disinfected with 5% bleach between uses.

F. At the beginning of the experiment, animals will be individually infected with *Batrachochytrium dendrobatidis* (*Bd*) by exposing an individual to 10^7 zoospores of *Bd* in 10 ml of H2O in a 90 mm petri dish or 50 ml conical centrifuge tube for 6-8 hours.

G. Animals will be swabbed periodically throughout the experiment with dry swabs (Medical Wire and Equipment) on the ventrum near abdomen and by 5 sweeps on the skin between each of the limb digits.

D.4.4. Procedures for Water Conditioning, Cage Set-up and Water Changes

A. Fill a 5 gallon bucket with deionized water from the sink.

B. Add water conditioner according to the bottle instructions (1ml per 5 gallons of water). **Do not use unconditioned water to fill aquaria.**

C. Allow the water to equilibrate to room temperature for 24 hours prior to use.

D. Check the pH of the water in one treatment once weekly using a pH test strip.

E. Record pH on the room sheet. Report a pH > 8 or < 6 to the area husbandry supervisor and the veterinary resident.

F. Conduct comprehensive water testing weekly in a sample of aquaria prior to water change with a Mardel Master Test Kit Strip or other appropriate testing kit.

G. Report levels above normal (ammonia >0.5mg/l, nitrite >0.5mg/l, nitrate > 50mg/l respectively) to the project PI.

H. Water will be adjusted to pH 6.5-7.5 by adding an aquarium buffer as necessary before water changes, and will be replaced more frequently if ammonia, nitrite or nitrate concentration reach abnormal levels.

I. If levels are abnormal, collect 10 milliliters of original water into a syringe or small receptacle. Give water to a member of the veterinary staff for possible retesting.

J. Record water quality levels on the animal room sheet.

D.4.5. Housing Frogs

A. After initial quarantine (see 8. Newly Received Frog Procedures below), the animals will be separated into multiple 10 gallon aquaria containing up to 10 individuals each.

B. House animals in at least 1 gallon of water per 4 individuals.

C. Animals will be housed in aquaria with sterilized terra-cotta pots, PVC pipes, aquarium gravel, and artificial aquatic plants to provide cover and enrichment.

D. Fluorescent lighting on a 12 hr cycle will be used in the animal care room, and the room temperature will be set to 68°F.

E. After infection with *Bd*, single individuals will be housed in quart-sized plastic cages filled with conditioned water. Plastic cages will also contain gravel and PVC pipe sections as enrichment. Animals will be monitored over the course of the infection, and the water changed weekly.

Alternatively, some experimental procedures will continue housing infected animals in groups as described above (A, B, and C).

D.4.6. Water Changes

A. Half the volume of water in each aquarium will be changed weekly.

B. When water change occurs on a feeding day, wait three hours after feeding to change the water.

C. Used water will be treated with 5% bleach before disposal to prevent pathogen discharge into the environment.

D.4.7. Equipment Change and Sanitation Schedule

A. The same aquaria and nets will be used for the duration of the experiment.

B. Aquaria, nets, and other husbandry tools will be disinfected with 5% bleach between uses (described above in 3. Procedures for Handling Frogs)

C. Animal care room will be swept and mopped weekly.

D. All aquaria will be disinfected with 5% bleach at the end of the experiment.

D.4.8. Newly Received Frog Procedures

A. Adult dwarf clawed frogs (*Hymenochirus curtipes*) will be obtained from an aquarium supplier such as Segrest Farms or Live Aquaria.

B. Visually inspect incoming animals for evidence of disease and submit an ATR if any abnormalities are observed.

C. Gradually expose frogs to ULAM conditioned water.

D. Remove water from the vendors shipping container so that it is approximately half full.

E. Add conditioned, room temperature water from ULAM in a volume equal to that already in the shipping container.

F. Allow the water in the shipping container to reach room temperature (~1 hour) before placing the frogs into clean ULAM aquaria.

G. Quarantine up to 20 incoming frogs in a heated, 10 gallon glass aquarium to clear any fungal infection.

H. Slowly heat (over 5-7 days) quarantine aquaria to 86°F using an aquarium heater, and maintain a water temperature of at least 86°F for 7 days to clear incoming fungal infection before moving animals to permanent tanks (described in 5. Housing Frogs above).

D.4.9. Death

A. Report unexplained deaths (i.e., those not due to experimental infection) during daily health checks to veterinary personnel. Refer to the ULAM Dead Animal Reporting SOP.

B. At 25 weeks post-inoculation, cultures of chytrid will be obtained from a sample of individual frogs per population. The frogs will be euthanized by decapitation with surgical scissors by a cut posterior to the eyes followed by double pithing of the brain and spinal cord with a pithing rod.

Using the euthanized frogs, cultures will be obtained from interdigital skin of the hindlimbs removed using surgical scissors or a biopsy punch.

C. The remaining frogs at the end of the experiment will be euthanized using pharmaceutical grade MS-222 as described above (1. Feeding and Health Checks).

D.4.10. Procedures for Adverse Events or Emergencies

A. In the case of adverse events or a disaster such as power failure, temperature issues, reduced operations in an adverse weather situation or other University closures, husbandry personnel should contact the project PI (Timothy James) or Ph.D. Student leading the experiment (Thomas Jenkinson) using contact information listed at the beginning of this SOP to ensure that animals will continue receiving daily health checks and feeding. For other emergencies contact University Department of Public Safety (734-763-1131 or dial 911).

D.5 Related Documents

1. ULAM Animal Care Identification and Communication Procedures
2. ULAM Dead Animal Reporting SOP

Appendix E

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