Lineage Composition and Genetic Diversity of *Batrachochytrium dendrobatidis* in the California Bay Area

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ABSTRACT

Chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis (Bd)*, has caused the decline and extinction of over 500 species of amphibians. Different lineages of the fungus are found throughout the world due to the human-mediated transportation and trade of amphibians. While *Bd*-GPL – a hypervirulent lineage – is found across the globe, other lineages are regionally endemic. Newly introduced and rapidly spreading lineages typically have low genetic diversity because they do not have sufficient time to diversify. In contrast, lineages that have existed in an area for a long time exhibit more diversity due to a history of host-pathogen coevolution. In California, *Bd*-GPL is the only lineage known to occur, yet studies have not yet characterized the fungus's genetic diversity in areas that exhibit a high flux of incoming amphibians. To discover the lineage composition and genetic diversity of *Bd* in the San Francisco Bay Area, I sequenced 240 genes from the *Bd* genome infecting 4 host species collected over 5 years, then constructed a maximum likelihood phylogeny showing which samples were most closely related. Among the samples that grouped with known *Bd*-GPL references, there was no association between *Bd* genetics and species or year, suggesting that the fungus is spreading quickly across the study area. Additionally, 3 samples grouped with known standards of the South American lineage *Bd*-Brazil, providing evidence that this lineage has now entered California. My study shows that human-mediated transportation has the power to bring diseases to new areas, allowing them to interact with communities in new ways.

KEYWORDS

chytridiomycosis, disease ecology, invasive species, amphibians, emerging infectious diseases

INTRODUCTION

Batrachochytrium dendrobatidis (*Bd*) causes the infectious disease chytridiomycosis found in amphibians worldwide (Longcore et al. 1999). The widespread prevalence of *Bd* may be attributed to the transportation and trade of amphibians resulting in the introduction of non-native infected animals to local amphibian communities (Fisher and Garner 2007, Yap et al. 2015). Amphibians are infected by fungal zoospores that spread through water or by skin-to-skin contact (Johnson and Speare 2005). Once an organism is infected, the fungus degrades proteins in the extracellular matrix of the superficial epidermis, or outer layer of skin. As infection proceeds, lower layers of the epidermis become colonized and destroyed (Moss et al. 2010). Because the skin is essential for amphibians in maintaining homeostasis, chytrid has caused population declines and extinctions of over 500 species of amphibians (Scheele et al. 2019). This decline is the greatest loss of vertebrate biodiversity in recorded history (Skerratt et al. 2007, Scheele et al. 2019).

Bd is a diverse fungus containing several lineages spread across five continents. The most widely spread lineage *Bd*-GPL ("Global Panzootic Lineage") is a hypervirulent and recombinant lineage. It is found across five continents and is credited with the most severe global chytridiomycosis outbreaks (Farrer et al. 2011). *Bd*-Brazil, *Bd*-Cape, and *Bd*-Korea are highly divergent from *Bd*-GPL and vary by location, prevalence, and virulence (Becker et al. 2017). Genotypic variation in *Bd* lineages occurs because the lineages can undergo rapid genomic rearrangements. Therefore, lineages can evolve rapidly in response to selective pressure imposed by hosts and the environment (Farrer et al. 2013). Because of this host-pathogen relationship, amphibians have adapted to the local fungus over long periods of time, possibly explaining the decreased virulence of local strains (Farrer et al. 2013). In contrast, when newly introduced strains spread quickly into a new area, the overall genome diversity decreases, as the strains do not have time to evolve within populations before spreading (Morehouse et al. 2003, James et al. 2009).

Due to selective pressure, newly introduced strains have proven to be more virulent than endemic ones, which may explain the higher virulence and spread of *Bd*-GPL in areas where it is newly introduced (Becker et al. 2017). Rapid spread may cause a higher virulence because frogs have not had time to co-evolve and develop resilience. In Brazil, hosts are more resistant to the

local *Bd*-Brazil, which has persisted in the area far longer than *Bd*-GPL. Thus, *Bd*-GPL infects frogs at a far greater rate and can spread farther and more quickly (Jenkinson et al. 2016). However, in Korea, local strains are present at a far greater rate than Bd-GPL. Host-pathogen coevolution may allow native strains to out-compete introduced Bd-GPL (Bataille et al. 2013). The contrasting hypotheses on the behavior of native and introduced strains illuminates the need to learn how strains on different species behave in parallel in other areas of the world, such as the United States.

Frog declines and occurrences of *Bd* have been recently documented in California (Vredenburg et al. 2013). The introduction of the invasive North American bullfrog (*Rana catesbeiana*) carrying *Bd*-GPL to California may have resulted in native frog population declines from the introduction of the new fungal variant (Yap et al. 2018). The presence of the chytrid-resistant species *Pseudacris regilla* may have also played a role in the spread of *Bd. Pseudacris regilla* is a reservoir species, meaning that it is able to carry high loads of *Bd* without being harmed. Therefore, in areas in which it coexists with frog species susceptible to the fungus, it can pass on the disease to these frogs (Reeder et al. 2012). This ability of reservoir species is one of the leading causes of *Bd*'s high pathogenicity (Valenzuela-Sanchez et al. 2017). Although *Bd* exists in California and native species, including *Bufo boreas* and *Rana draytonii,* have been affected, we have yet to understand how *Bd* on different species interact with one another and whether *Bd* is spreading as an epidemic or evolving with the host population.

The key to understanding these questions lies in elucidating the genetic diversity of disease strains infecting these species in the Bay Area. Sites in this area have been sampled for the presence of *Bd* on local amphibians (Stutz et al. 2017). All four species sampled, *B. boreas, P. regilla, R. catesbeiana,* and *R. draytonii*, had individuals carrying *Bd* over the years 2013 to 2017. However, it is currently unknown what lineages are present, and what species and temporal relationships exist with the hosts. This study aims to characterize the genetic diversity of *Bd* in California frogs and to determine how this diversity varies across species and time. Determining this variation will provide information on how *Bd* spreads between species. My goals are to: (1) determine what *Bd* lineages are present in the Bay Area, (2) determine how the genetic diversity of *Bd* varies across species, and (3) identify how the overall genetic diversity changes over time.

METHODS

Study site

To understand genetic variation of *Bd* across species and time, I used samples collected from sites located in the East Bay Area of California, from Alameda and Santa Clara counties (Figure 1). These counties share a similar climate, characterized as warm-summer Mediterranean, with warm, dry summers, and mild, wet winters.

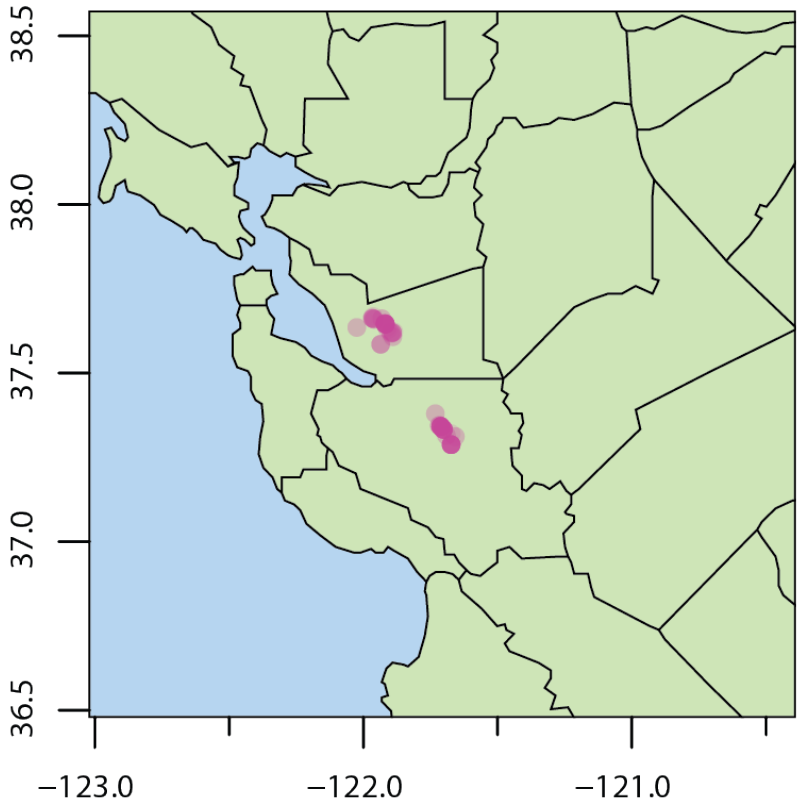

Figure 1. Distribution of collected samples. Samples were collected from Alameda and Santa Clara counties by the Briggs Lab, UCSB.

Sample collection

To collect DNA for genetic analysis, I obtained samples from 143 California frog skin swabs. These samples were collected by the Briggs Laboratory at the University of California, Santa Barbara. The Briggs Laboratory recorded their DNA samples with the collection year,

collection location, host frog species, and *Bd* load (measured in zoospore equivalents). I chose samples with high loads of *Bd* (greater than 50 zoospore equivalents, if available) and maximized across species and year (Table 1). I included all four species sampled by the Briggs Lab: *B. boreas* (BUBO), *P. regilla* (PSRE), *R. catesbeiana* (RACA), and *R. draytonii* (RADR) (Table 2).

Table 1: Counts of samples by species and year.

	2013	2014	2015	2016	2017	Total
BUBO	3	5	12	13	10	43
PSRE	8	5	5	11	15	44
RACA	1	1	4	13	7	26
RADR	6	4	7	5	8	30
Total	18	15	28	42	40	143

Table 2: Sampled species.

Species	Natural History	Invasive?	Affected by *Bd*?
Bufo boreas Western toad (BUBO)	Native to western North America, encountered during the wet season near roads, or otherwise near water (Frost 2018).	No	Moderately
Pseudacris regilla Pacific chorus frog (PSRE)	Found sea level to 10000+ ft elevation, reproduce in aquatic settings, grows up to 2 in length (Frost 2018).	No	No
Rana catesbeiana American bullfrog (RACA)	Inhabits permanent water bodies, relatively immune to chytrid, spreads the disease as it invades new areas (Frost 2018).	Yes	Slightly
Rana draytonii California red-legged frog (RADR)	Endemic to CA, IUCN vulnerable species, federally listed threatened species, found in coast ranges and Sierra Nevada foothills (Frost 2018).	No	Highly

DNA extraction and genotyping

To amplify sequences with sufficient pathogen DNA, I first used a precipitation protocol to purify DNA (Appendix A: Precipitation Protocol). Once DNA was suspended, I prepared the samples for pre-amplification of 240 pre-selected genes of *Bd* that are known to vary across lineages and isolates (Appendix A: PCR Protocol). To enrich the DNA template of the target regions, I performed the pre-amplification reactions according to established protocol (Byrne et

al. 2016). Following the reaction cycle, I cleaned the products using an ExoSAP dilution (Appendix A: ExoSAP Protocol). I then pooled the forward and reverse products and sent the products to the University of Idaho IBEST Genomics Resources Core for multiplex PCR using the Fluidigm Access Array chip and subsequent Illumina MiSeq sequencing.

Sequence Analysis

I obtained raw sequence data from the University of Idaho laboratory and generated a consensus sequence for each sample as described in Byrne et al. (2016). The consensus sequence is a single sequence composed of the most frequent bases found at each position. To filter genotypes on length and quality, I used custom R scripts generated by the Rosenblum Laboratory (University of California, Berkeley). Using these scripts, I also created a data set of the loci of each sample and aligned the loci separately. I concatenated each locus, then aligned consensus sequences using MUSCLE v. 3.24 (Edgar 2004).

To visually inspect sequences and check for errors following alignment, I used Geneious Prime v. 2019.0.4 (https://www.geneious.com). In Geneious Prime, I created a tree using the RAxML plug-in v.8.2 (Stamatakis 2014), and the accepted maximum likelihood algorithm (GTR+Gamma+I) with 100 bootstrap replicates. The maximum likelihood model generated a preferred tree based on the likelihood of sequence evolution, with the generated tree indicating the most plausible evolutionary relationships between pathogen strains. To compare experimental sequences with known ones, I included known *Bd* sequences from the Rosenblum Laboratory. The concatenated phylogenetic tree was plotted using FigTree v1.4.4 (Rambaut 2018).

I calculated genetic diversity using a heterozygosity test for diversity (H_0), with higher values indicating more divergence. To calculate H_0 values for each sample, I used the consensus alignment generated previously and custom scripts in Python v. 3.7.3 (Python Software Foundation 2019). I also grouped the samples by host species and year and calculated the mean H_0 for each group using R v. 3.5.1 (R Core Team 2018). Using a Dunn's Test from the R package FSA v0.8.23 (Ogle et al. 2019), I confirmed my data was normally distributed. I then tested for association between mean heterozygosity and host species or year with a one-way ANOVA test in R.

RESULTS

Amplification success

Of the 143 samples I sent to the sequencing laboratory, 48 returned with usable data, resulting in a success rate of 33.8%. All four species and all five years were represented (Table 3).

Table 3. Samples used in phylogenetic and heterozygosity analyses.

	2013	2014	2015	2016	2017	Total
BUBO	2	2	2	4	6	16
PSRE	3	4	2	5	7	21
RACA	0	0	0	1	3	4
RADR	2	1	1	2	1	7
Total	7	7	5	12	17	48

Phylogenetic analysis

Phylogenetic analysis of the samples showed that *Bd* was distributed throughout the tree, without clustering based on host species (Figure 2). I found that *Bd* present on resistant species and *Bd* present on susceptible species did not cluster separately on the tree. Similarly, *Bd* present on invasive species and *Bd* present on native species also did not cluster separately. I also did not find a temporal trend on the phylogenetic tree, as years were distributed throughout the tree and did not group together (Figure 3).

Three of the samples grouped with sequences from known *Bd*-Brazil isolates (*Bd*_LFT001_10 and *Bd*_UM142). All three samples were from the same host species (*B. boreas)*, the same year (2017), and the same sampling site. All other samples grouped with the known *Bd*-GPL isolates.

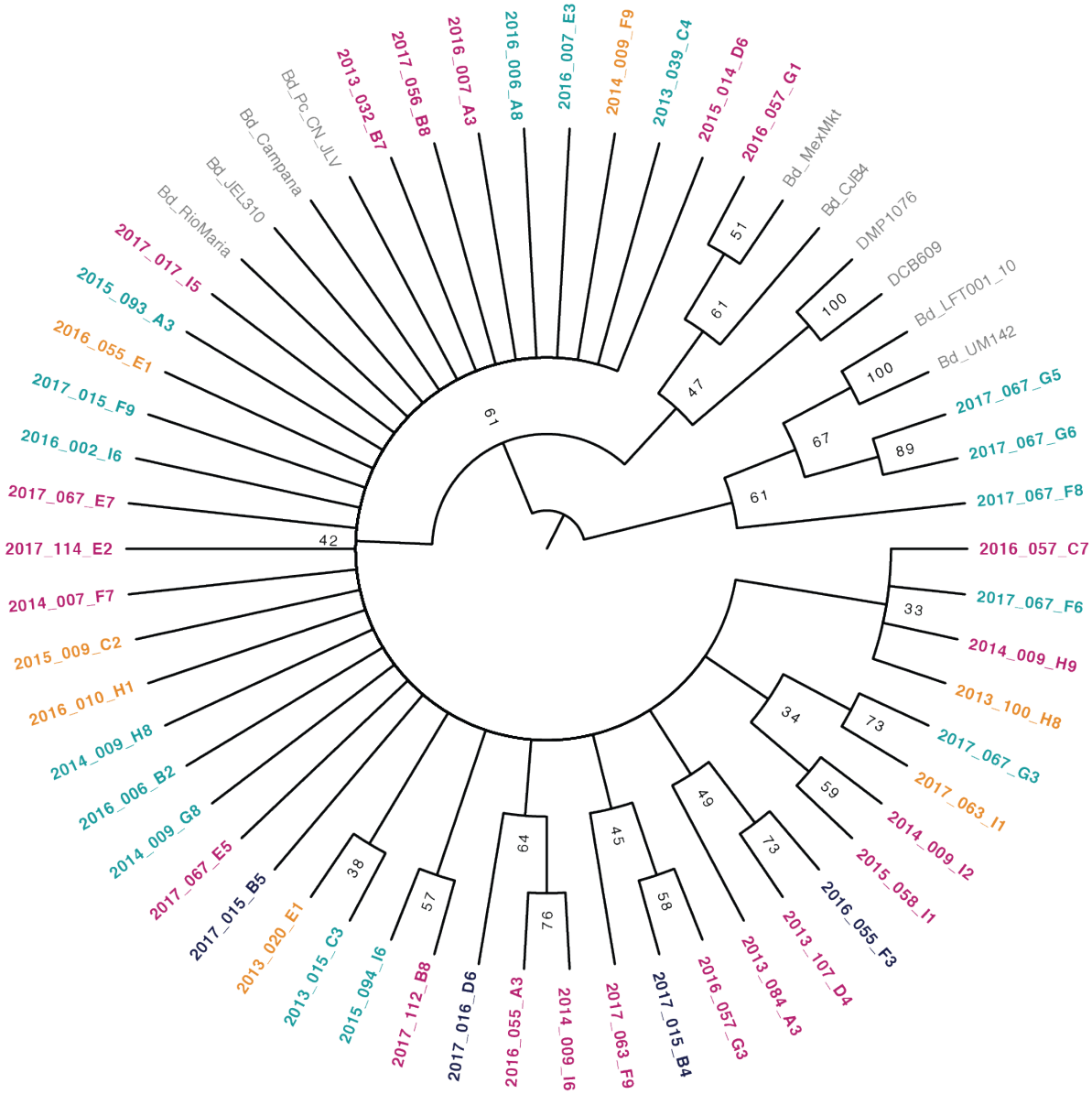

Figure 2. Maximum likelihood tree of all samples, labeled by species. Key: Teal = BUBO, Pink = PSRE, Dark blue = RACA, Yellow = RADR, Grey = reference isolates.

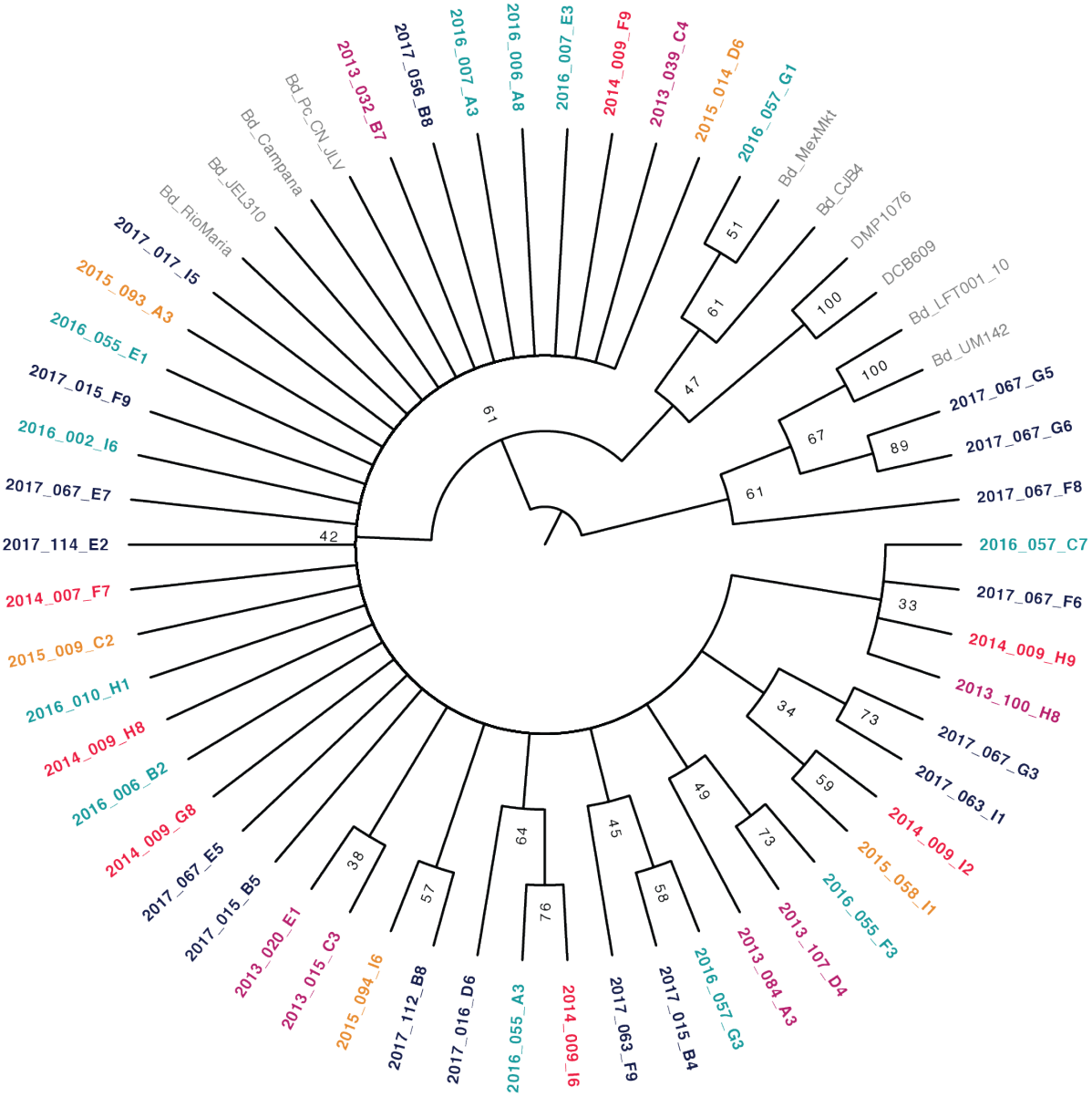

Figure 3. Maximum likelihood tree of all samples, labeled by year. Key: Pink = 2013, Red = 2014, =Yellow = 2015, Teal = 2016, Dark blue = 2017, Grey = reference isolates

Heterozygosity testing

After conducting a heterozygosity test, I found no correlation between heterozygosity and either host species or time (Figure 4, Table 4).

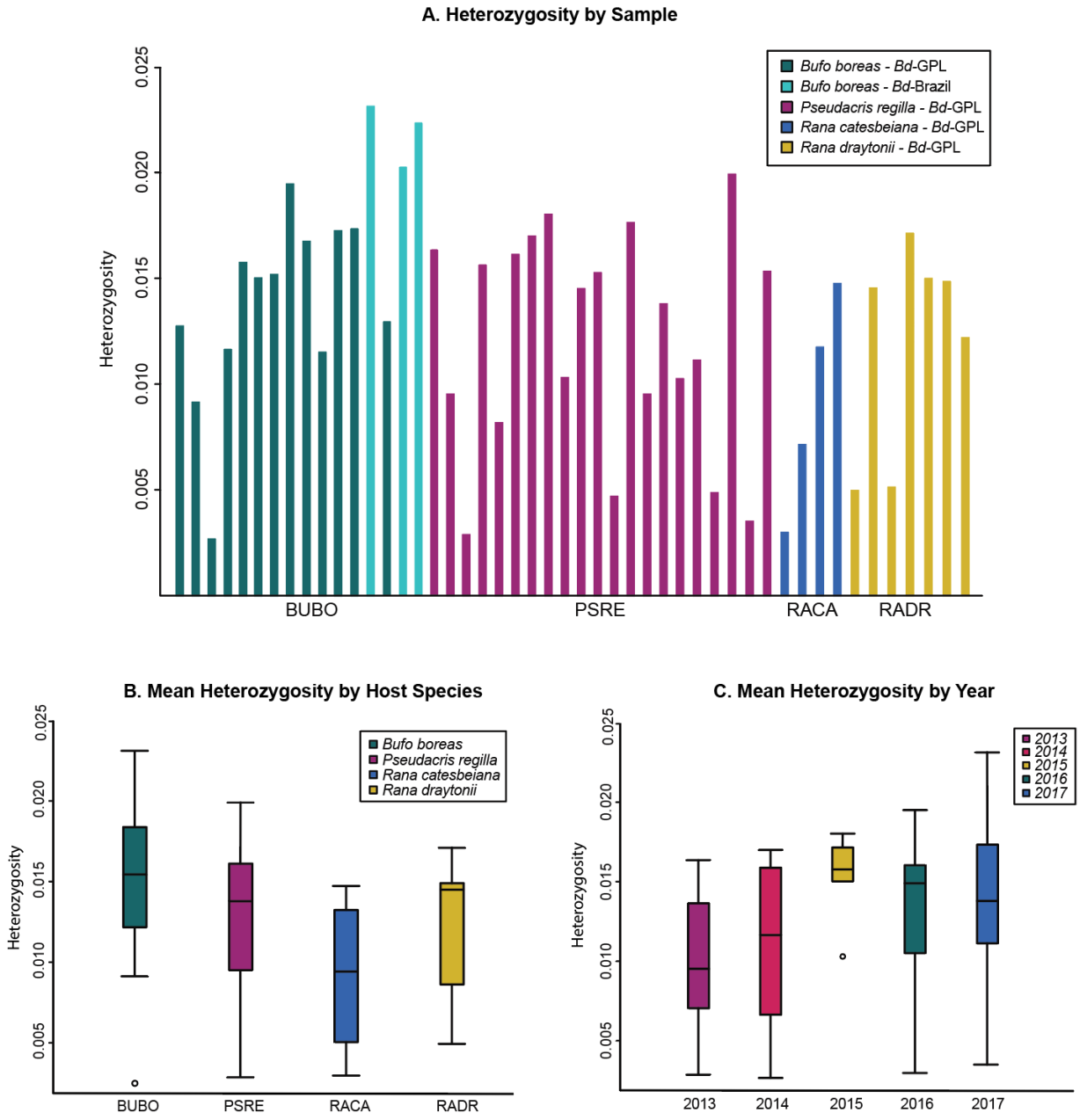

Figure 4. Heterozygosity values. A. Heterozygosity values for each sample, colored by host species. B. Mean heterozygosity for each host species. C. Mean heterozygosity for each year.

Table 4. ANOVA test for association between heterozygosity and factors.

Factor	F value	Df	P value
Species	2.108	3	0.114
Year	1.465	4	0.231

Heterozygosity values were not significantly different between species groups. The highest H_0 values were found in the three samples that grouped with *Bd*-Brazil, though the difference was not statistically significant. Earlier years had lower H_0 values than later years, but this difference was also not statistically significant.

DISCUSSION

My results showed that two lineages of *Bd* exist in California, *Bd*-GPL and *Bd*-Brazil. *Bd*-GPL – the global hypervirulent lineage – has been known to exist throughout California, but *Bd*-Brazil is a novel lineage in the state. *Bd*-GPL's low host specificity can account for the spread of species and years on the phylogenetic tree, with no clustering of samples from the same species or year group. This low host specificity indicates that *Bd* is spreading quickly between members of different species. The diversity of species in the environment and the presence of anthropogenically introduced invasive species can account for both the presence of the novel lineage and the epidemic-like spread of *Bd* in the Bay Area.

How genetically diverse is *Bd* in California?

My discovery of *Bd*-Brazil in California supports the hypothesis that continued anthropogenic introductions of invasive disease lineages are furthering the global spread of *Bd.* With the first report of this lineage in California, I genetically demonstrate that *Bd* is arriving in California from multiple source populations. There have been other cases in which geographic isolation has been overcome by human introduction. In islands off the coast of California, including the Channel Islands and San Francisco Bay Islands, *Bd*-GPL was found to emerge following movement of humans to the islands (Yap et al. 2015). Likewise, the Bay Area is

completely separated from Brazil geographically yet connected anthropogenically. The Bay Area is home to the Ports of San Francisco and Oakland, and San Francisco and Oakland International Airports. Researchers estimate almost 10 million bullfrogs entered through the port of San Francisco between 2000 and 2005, and it is likely the incoming flux of this species has increased since (Schloegel et al. 2009). Through these locations, introduced disease-carrying bullfrogs (*R. catesbeiana)* – which comprise 99% of incoming amphibians – can readily enter California and transmit novel lineages to native species.

Bd-GPL is the most widely studied and understood variant of *Bd* given that it was discovered in 1998. On the other hand, very little is known about the *Bd*-Brazil lineage because it was only discovered in 2012. In Brazil, *Bd*-Brazil has existed in the ecosystem longer than the invasive *Bd*-GPL, and amphibians exhibit fewer declines from the endemic lineage (Becker et al. 2017). However, while these two lineages differ genotypically, their phenotypes do not show such variation. It is suspected that host-pathogen coevolution – not phenotypic differences – has caused *Bd*-Brazil's lower virulence as compared to *Bd*-GPL. However, in areas that have not been exposed to the Brazilian endemic lineage, *Bd*-Brazil may pose a threat to amphibians (Becker et al. 2017). Because it is such a mysterious lineage, there is some disagreement about its genetic history, but we know it plays an important role in the Brazilian ecosystem. Given the differences in host-pathogen coevolution and uncertainty surrounding *Bd*-Brazil, we do not know how the Brazilian lineage will act in the California ecosystem. My sample size was small, and I only observed *Bd*-Brazil on a single species – *B. boreas*. However, if this pattern of infection is true in nature –if *Bd*-Brazil favors *B. boreas* – it can provide us with insight into the ecology of this mysterious lineage.

Bufo boreas from 2017 was the only species carrying *Bd*-Brazil, indicating that the lineage might be a recent introduction. Natural history characteristics of *B. boreas* may have contributed to this species carrying the lineage. *Bufo boreas* is a burrowing frog, meaning it finds shelter underground, unlike the other species in the study (Stebbins, 1915). While *Bd*-GPL is known to withstand harsh conditions and higher levels of UV radiation, other lineages such as *Bd*-Brazil are more successful in mild climates (James et al. 2015, Jenkinson et al. 2016). *Bufo boreas*'s burrowing nature may have created a refuge for *Bd*-Brazil to survive.

How is *Bd* spreading in the Bay Area?

My finding that the overall genetic structure of *Bd* in the Bay Area is not host species dependent and is not exhibiting diversification over time supports the epidemic, or novel pathogen, hypothesis. This hypothesis proposes that as *Bd* enters a new area, it spreads quickly, causing rapid die-off (Morehouse et al. 2003, Rachowicz et al. 2005, James et al. 2009). The lines of evidence that support this hypothesis are: low host specificity, and low levels of temporal diversification of the fungus (Morehouse et al. 2003, James et al. 2009).

The spread of *Bd* can be understood in terms of host-pathogen interactions. While overall host diversity may not be an indicator of *Bd* success, the presence of specific species in an environment can affect disease dynamics (James et al. 2015). Resistant species such as *P. regilla* and *R. catesbeiana* can act as reservoirs by carrying very high loads of the fungus without dying off quickly (James et al. 2015). The reservoirs' ability to maintain high prevalence of *Bd* over long periods of time can – in turn – affect species that are highly sensitive to the fungus by passing virulent strains on to them. When these sensitive amphibians – such as *R. draytonii* and *B. boreas* – are exposed to *Bd* carried by reservoir species, they can contract the fungus quickly and die shortly after (James et al. 2015). During this short time span, the fungus does not have time to diversify. Because the accumulation of mutations takes time, the genetic pattern we observe fits a rapidly spreading pathogen.

Bd evolves through chromosomal copy variation and varying heterozygosity (Farrer et al. 2013, Rosenblum et al. 2013). Significant genetic differences accumulate in laboratory *Bd* strains on the timescale of approximately 30 generations, or 6 years (Langhammer et al. 2013, Refsnider et al. 2015). Therefore, we expect a long-term lineage to have accumulated more heterozygous mutations over time. Because my study included samples from 5 years, it is likely that, if *Bd* were experiencing genetic differentiation, patterns would have emerged on the phylogenetic tree and in the heterozygosity analysis. We may have observed clustering on the tree based on collection year, and an increasing pattern of heterozygosity in later years. However, because we did not see evidence of genetic divergence in either test, fungus may not be diversifying over time. The overall temporal homogeneity of the disease genetics provides further evidence that the novel pathogen hypothesis applies in the Bay Area.

Human introduction has facilitated *Bd* transfer through populations worldwide (Fisher and Garner 2007, Yap et al. 2015). The human trade of amphibians is what allows invasive species to enter new areas and bring in novel lineages (Fisher and Garner 2007, O'Hanlon et al. 2018). For instance, *Rana catesbeiana* has acted as a vector of *Bd* in other areas of the western United States (Daszak et al. 2004, Miaud et al. 2016, Yap et al. 2018). Because we know such a high volume of bullfrogs enter the Bay Area every year, this species has the capacity to transmit new genetic variants of the fungus to Bay Area frogs. Pathogen genotypes from the invasive species (*R. catesbeiana*) were found throughout the tree, suggesting that if *Bd* is brought by *R. catesbeiana,* it can be transferred throughout the population (Figure 2). My genetic results are consistent with previous findings documenting the spread of *Bd* into the Bay Area, and that the transfer can happen quickly.

Our results showing low temporal diversity support the novel pathogen hypothesis (Morehouse et al. 2003, James et al. 2009). My temporal study provides evidence that this hypothesis demonstrates the dynamics of *Bd*-GPL in the Bay Area. However, what happens if *Bd*-Brazil establishes? Because *Bd*-Brazil is a newly introduced lineage, it can exhibit this type of quick spread from the vector species to those that are endemic. Furthermore, it may have the potential to hybridize with *Bd*-GPL strains and produce a new hybrid lineage (Jenkinson et al. 2016). If *Bd*-Brazil persists and succeeds in this new area of the globe, it has the potential to quickly infect amphibians that have not previously been exposed and interact with these organisms in new ways.

Limitations

Limitations to the study included the scope of samples studied and the accuracy of analytic instruments. The initial sample size was small, consisting of 143 DNA samples. The initial counts of *R. catesbeiana* were lower than counts for the other species, comprising 26 of the 143 samples, despite being an important species to study. While it is likely that the species is the source of *Bd*-Brazil introduction, not enough species were included in the sample set to find evidence of the connection. Also, as only 5 years were studied, this could limit the conclusions drawn about temporal trends. Furthermore, Fluidigm sequencing returns detailed base pair

information at the expense of often losing some data. Because input DNA was not sufficient for sequencing, roughly 2/3 of the original dataset was unusable due to poor quality data.

Future Directions

Further research into the introduction of *Bd*-Brazil into California should have greater spatial replication, larger sample sizes, and longer time scales. More Bay Area frogs should be sampled, with a focus on invasive bullfrogs to determine whether this species is facilitating the introduction of *Bd* into the Bay Area. Swabbing animals at entry points, such as at docks and airports, may allow us to both determine how new lineages are entering California and provide direction for policy makers. It is also possible that *Bd*-Brazil arrived in the Bay Area following the introduction in a different California location. Therefore, sampling in other areas of California can show if this lineage now exists outside of the Bay Area, suggesting that *Bd* is moving throughout the state as well. Lastly, looking at *Bd* genomics on a longer timescale by including more samples from the years focused on in this study, as well as collecting samples into the future will allow us to see the history and impact of the new *Bd*-Brazil lineage. While my findings imply that *Bd*-Brazil is a recent introduction in the Bay Area, that invasive bullfrogs are a possible source of new genetic structure, and that *Bd*-GPL in the Bay Area is spreading quickly, we still need to determine *Bd*-Brazil's source and determine its future in California to mitigate possible negative consequences of its spread.

Broader Implications

The introduction of invasive species has a large impact on the survival of *Bd*-affected amphibians in California. As *Bd* spreads between species, novel introductions will affect native populations and contribute further to decline of already-threatened endangered species. The introduction of new lineages presents a new problem for conservation because there is so much uncertainty in how it will behave. History has shown the potential for devastation from the introduction of a novel disease strain, especially fungal diseases. For example, the emergence of the fungal diseases white nose syndrome in bats and meningitis in humans illustrate how these diseases have destructive potential to both humans and wildlife. We do not know how

detrimental *Bd*-Brazil will be in California, but if this new lineage reaches the epidemic levels seen in *Bd*-GPL, it has the potential to cause large-scale declines in native amphibian species – and we do not want to wait to find out. Although my research proposes potential mechanisms of the spread of new *Bd* lineages, further work will allow us to more robustly safeguard the state from deadly fungal disease outbreaks.

ACKNOWLEDGEMENTS

I thank my mentor Thomas Jenkinson for his continued support throughout the project, from helping me develop my research project, to teaching me how to run PCRs and handle genetic data. Other members of the Rosenblum Lab, Allison Byrne and Andrew Rothstein, were so helpful in getting me through the coding and bioinformatics part of the project. I also thank Erica Bree Rosenblum for giving me the opportunity to be a part of her amazing lab and giving me insight and direction for my post-grad future. Cheryl Briggs of the Briggs Lab at the University of California, Santa Barbara was essential in supplying the DNA field swab samples I analyzed for the project. Patina Mendez and Leslie McGinnis were such a key part of the process, by giving feedback on my writing and advice during office hours. Finally, I benefited greatly from the advice, support, and inspiration from my peer writing group, Victoria Glynn, Christopher Orner, and Madeleine Zuercher.

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APPENDIX A: Protocols

Precipitation protocol

1. Place 70% ethanol in freezer to chill
2. Bring DNA sample to 200 µL with low TE (estimate sample volume beforehand)
3. Premix low TE, NaOAc, and glycogen according to table:

Reagent	Per sample (µL)
Low TE	250
NaOAc	50
Glycogen	2.5
Total	302.5

4. Add 302.5 µL of premix from step 2 to each sample
5. Add 500 µL isopropanol, invert 10x
6. Put samples in fridge overnight (approximately 24 hrs).
7. Spin in centrifuge at 13,000x g for 10 min (rcf), if no pellet, repeat
8. Pull off supernatant with P1000 and p200 micropipettes
9. Wash with 500 µL cold 70% ethanol
10. Flick and invert tube to dislodge pellet
11. Let sit at room temperature for 5 min
12. Spin 1 min at 13,000x g
13. Pull off ethanol with P1000, P200, and P10 pipettes and let dry
14. Resuspend pellet in 20 µL low TE
15. Let pellet resuspend in fridge overnight

PCR Protocol

1. Prepare reagents for master mix; put everything on ice except DMSO:

Reagent	Per Sample (µL)
10x MgCl	1
25mM MgCl	1.08

DMSO	0.5
10mM dNTPs	0.2
Primer Pool (A or B)	1.4
H2O	4.62
Faststart Taq (leave in freezer until ready)	0.2

2. Combine reagents: one tube with forward primer, one with reverse primer
3. Dispense master mix into 8 strip tubes (total volume/8)
4. Dispense 9 µL mix into each well of PCR plate
5. Flick and centrifuge each DNA sample, then dispense 1 µL into corresponding well of PCR plate
6. Plate centrifuge on short for total 20 seconds
7. Run PCR cycle

ExoSAP Clean Up Protocol

1. Make 1:5 dilution exosap:water for a total of 405 µL
2. Dispense 50 µL in each well of 8 strip tubes
3. Dispense 4 µL per well of PCR plate
4. Cap PCR plate and centrifuge on short for total of 20 seconds
5. In a new PCR plate, dilute exosap product by dispensing 5 µL product and 13 µL H2O
6. Plate centrifuge on short for 20 seconds
7. In a third PCR plate, dispense 5 µL from each pool (A and B) into new plate. (i.e. mix the A and B product corresponding to each DNA sample)
8. Cap plates, and send the plate with the combined product off for sequencing