BATRACHOCHYTRIUM DENDROBATIDIS (BD) PREVALENCE: AN ANALYSIS OF THE *ATELOPUS* SPECIES POPULATION DECLINE IN PANAMA AND COSTA RICA

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Diana Esperanza

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Diana Esperanza

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by

____

John Withey, Ph. D.

Member of the Faculty

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ABSTRACT

Batrachochytrium Dendrobatidis (Bd) Prevalence: An Analysis of the *Atelopus* Species Population Decline in Panama and Costa Rica

Diana Esperanza

The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd) has caused amphibian declines and extinctions around the world. Bd has spread in Costa Rica throughout Panama and has caused some populations of the endemic *Atelopus* genus to decline and some species to become extinct. In this thesis, I addressed the life history, and ecological traits of *Atelopus* species of Costa Rica and Panama using data from La Marca et al. (2005) and the International Union for Conservation of Nature's Red List of Threatened Species (IUCN) to determine the connection to their population decline and extinction. Then, I addressed the connection of the *Atelopus* species genetics with population decline and extinction using DNA sequencing from 5 *Atelopus* species of Costa Rica and Panama. Lastly, I addressed the connection between Bd prevalence and bioclimatic variables in *Atelopus* species of Panama and Costa Rica. The results revealed that (1) a pattern of extinction in *Atelopus* species that occurred at the highest elevation in isolated geographic areas. (2) the *Atelopus* species vulnerability to population decline and extinction may have been caused by their geographic isolation resulting in weaker immune system. (3) Bioclimatic variables could have contributed to Bd prevalence that caused extinction or population decline in the *Atelopus* species of Costa Rica and Panama due to patterns of low temperature and high precipitation. The results suggest that environmental factors and the *Atelopus* response to disease may have contributed to their population decline and extinction.

Table of Contents

CHAPTER 1 Introduction ***1***

1.1 General Pattern of Amphibian Extinction 1

1.2 Positionality Statement 2

CHAPTER 2 Literature Review ***4***

2.1 Introduction 4

2.2 The Importance of Amphibian Species 6

2.3 Life History and Transmission of Bd 7

2.4 Pathology of Bd 8

2.5 Origin of *Batrachochytrium dendrobatidis* (Bd) and the Cause of its Spread 11

2.6 How did Bd Spread Around the World? 12

2.7 Major Trends in Amphibian Extinctions 13

2.8 The Harlequin Frogs (Genus *Atelopus*) of Panama and Costa Rica 13

2.9 Detailed history of Bd and Amphibian Extinction in Panama 16

2.10 Bd Spread Across the East of the Panama Canal 18

2.11 Is there a Relationship between Bd and Climate Change? 20

2.12 Panamanian Frogs Face Conservation Challenges 23

2.13 Are Amphibians in Panama Surviving Bd? 26

2.14 The Effect of Ecological Variables in Amphibians' Skin Defenses Against Bd 27

2.15 Reintroduction of the *Atelopus limosus* into the Wild 29

2.16 Conclusion 30

CHAPTER 3 Methods ***33***

3.1 Forensic History of the *Atelopus* Species in Costa Rica and Panama 33

3.2 *Atelopus* Species Phylogenetic Tree 33

3.3 Correlation Between Bd Prevalence in *Atelopus* Species and Climatic Variables in Panama and Costa Rica 34

CHAPTER 4 Results ***36***

4.1 Forensic History of the *Atelopus* species in Costa Rica and Panama 36

4.1.1 Chiriquí Harlequin Frog (*Atelopus chiriquiensis)* 36

4.1.2 Variable Harlequin Frog *(Atelopus varius)* 38

4.1.3 Limosa Harlequin Frog *(Atelopus limosus)* 42

4.1.4 Panamanian Golden Frog *(Atelopus zeteki)* 44

4.1.5 Pirre Harlequin Frog *(Atelopus glyphus)* 47

4.1.6 The Darien Stubfoot Toad *(Atelopus certus)* 49

4.1.7 The Chirripó Stubfoot Toad *(Atelopus chirripoensis)* 51

4.1.8 Pass Stubfoot Toad *(Atelopus senex)* 53

4.2 *Atelopus* Species Phylogenetic Tree 55

4.3 Connection Between Bd Prevalence and Bioclimatic Variables in *Atelopus* Species of Panama and Costa Rica 57

4.3.1 Chiriquí Harlequin Frog *(Atelopus chiriquiensis)* 58

4.3.2 Variable Harlequin Frog *(Atelopus varius)* 61

4.3.3 Darien Stubfoot Toad *(Atelopus certus)* 64

4.3.4 Pass Stubfoot Toad *(Atelopus senex)* 66

CHAPTER 5 Discussion 70

5.1 Discussion 70

5.2 Limitations of the Study 72

5.4 Next Steps and Future Studies 74

5.5 Conclusion 74

Bibliography……… 77

List of Figures

Figure 1. Redness of the skin indicates the presence of Bd. 8
Figure 2. Zoosporangium releases zoospores 8
Figure 3. Bd lifecycle starts when spores burrow into the frog's skin. Source: Rosenblum et al. (2010). 9
Figure 4. Map of Bd detection from Mexico through Central America. Source: Cheng et al. (2011). 10
Figure 5. Distribution map for Atelopus species in Costa Rica and Panama. Species missing on this map: A. senex and A. chirripoensis. Source: Lewis et al. (2018)........... 15
Figure 6. Map of Costa Rica and west Panama with sites of amphibian declines. The lines mean date and location of the declines. Source: Lips et al. (2006). 17
Figure 7. Timeline of Bd Detection Throughout Costa Rica and Panama 20
Figure 8. Survival patterns of frogs treated with J lividum before Bd exposure and frogs exposed to Bd without treatment. 118 days after exposure the treated frogs were infected by Bd. J lividum bacteria was not present at the death of the treated frogs. Source: Becker et al., (2011). 25
Figure 9. Map of Panama and the four sites studied. Source: Varela et al. (2018). 28
Figure 10. Bacterial community composition in D. auratus across the sites near the Panama Canal. Varela et al. (2018) used linear discriminant analysis (LDA) scores to find the most significance in bacterial composition. Source: Varela et al. (2018).................. 29
Figure 11. The mean elevation of Costa Rica and Panama ranges from 600 m to 3450 m. 55
Figure 12. Atelopus Species Phylogenetic Tree and IUCN Red List Assessment 56
Figure 13. ggplot of BIO1 (left) with temp. range from 7°C to 20 °C per year and BIO12 (right) with precipitation. range from 2000 mm to 5000mm per year in Costa Rica and Panama. 57
Figure 14. Map of the Atelopus chiriquiensis Distribution in Costa Rica and Panama ... 58
Figure 15. Atelopus chiriquiensis Response to Bioclimatic variables range of values based on the estimates of the probability of occurrences. 60
Figure 16. Predicting Recent Climatic Habitat Suitability on the Atelopus chiriquiensis range.. 61
Figure 17. Map of the Atelopus varius Distribution in Costa Rica throughout Panama .. 61
Figure 18. Atelopus varius Response to Bioclimatic variables range of values based on the estimates of the probability of occurrences... 63
Figure 19. Predicting Recent Climatic Habitat Suitability of the Atelopus varius range . 63
Figure 20. Map of the Atelopus limosus Distribution in Panama.... **Error! Bookmark not defined.**
Figure 21. Atelopus limosus Response to Bioclimatic variables range of values based on the estimates of the probability of occurrences.................**Error! Bookmark not defined.**
Figure 22. Predicting Recent Climatic Habitat Suitability of the Atelopus limosus range ....**Error! Bookmark not defined.**
Figure 23. Map of the Atelopus zeteki Distribution in Panama ...... **Error! Bookmark not defined.**
Figure 24. Predicting Recent Climatic Habitat Suitability of the Atelopus zeteki range ....**Error! Bookmark not defined.**

Figure 25. Map of the Atelopus glyphus Distribution in Panama ... **Error! Bookmark not defined.**
Figure 26. Predicting Recent Climatic Habitat Suitability of the Atelopus glyphus range .... **Error! Bookmark not defined.**
Figure 27. Map of the Atelopus certus Distribution in Panama ...... **Error! Bookmark not defined.**
Figure 28. Atelopus certus Response to Bioclimatic variables range of values based on the estimates of the probability of occurrences.................. **Error! Bookmark not defined.**
Figure 29. Predicting Recent Bioclimatic Habitat Suitability of the Atelopus certus range. 66
Figure 30. Map of the Atelopus chirripoensis Distribution in Costa Rica................ **Error! Bookmark not defined.**
Figure 31. Map of the Atelopus senex Distribution in Costa Rica 66
Figure 32. Atelopus senex Response to Bioclimatic variables range of values based on the estimates of the probability of occurrences. 68
Figure 33. Predicting Recent Bioclimatic Habitat Suitability of the Atelopus senex range. 68

List of Tables

Table 1. IUCN 2019 assessment of the amphibian species by groups. From the 6,892 of amphibians assessed 6,098 species are frogs and toads, and 40% (2,409 species) are extinct or threatened. 13

Table 2. Amphibian decline in El Copé, Central Panama. Loss of diversity was measured by four indicators: named species, candidate species, lineages (named + candidate species), and PD (sum of branch lengths obtained by MPL analysis of phylogenetic tree). Source: Crawford et al. (2010). 18

Table 3. Atelopus zeteki infection intensity (number of zoospores on skin swabs) and zoospore output (number of zoospores released per minute) at death. Source: DiRenzo et al. (2014). 26

Table 4. Summary data on Atelopus species. Prot. areas: Bd presence in protected areas. Yr. of last record: year of most recent record; Yr. Bd presence of Bd: year(s) documented; Hab. destr: occurrence of significant habitat destruction/ Status: Stable, Decline* 54

Table 5. Summary of Location, Coordinates Minimum, Mean, and Maximum Elevation of Atelopus Species Occurrences. 55

Table 6. . List of 19 bioclimatic variables used in bioclimatic model development. Names and descriptions are in reference to the WorldClim, Hijmans, 2017. 58

Table 7. Summary of the Atelopus species' Probability of Occurrence in BIO1 and BIO12, and the Probability of Occurrence with Habitat Suitability 69

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CHAPTER 1 Introduction

1.1 General Pattern of Amphibian Extinction

Many factors can affect amphibian population declines. Amphibian species around the world are facing threats such as habitat loss, introduced invasive species, pollution and disease (Baillie et al., 2004). However, one major cause of amphibian declines is a skin infection called Chytridiomycosis. Chytridiomycosis is caused by the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd from this point on; Crawford et al., 2010). Bd has been spreading throughout Central American countries since at least the 1980's and has caused the loss of amphibian diversity.

Batrachochytrium dendrobatidis is a chytrid fungus that infects the skin of vertebrate species (Longcore et al., 1999). Bd grows in humid environments (Kirshtein et al., 2007) and low temperatures (Longcore et al., 1999), suggesting that amphibians living in high elevation habitats are most at risk of Bd infections (LaMarca et al., 2005).

The first detection of Bd in Central America was in the late 1980's and caused the extinction of the Costa Rican Golden toad *(Bufo periglenes)* (Baillie et al., 2004). Since 1993, Bd has been detected in the highlands of the Panamanian tropical rainforest, including Eastern Panama and across the Panama Canal (Rodriguez-Brenes et al., 2016). Bd is rapidly approaching Panamanian sites with minimal human occupation.

New research indicates that some amphibian species endemic to Panama may be resisting Bd through skin defenses. In 2014, nine amphibian species appeared to be recovering from Bd at the same location of the breakout a decade ago in 2004, including the harlequin frog *(Atelopus varius),* and the rocket frog *(Colostethus panamansis)*

(Voyles et al., 2018). These authors suggested that the abundance of amphibian skin microbiome may impact their vulnerability to Bd, and that evolution in this microbiome may have created a resistance to Bd (Voyles et al., 2018).

1.2 Positionality Statement

My thesis is intended to bring awareness of the loss of *Atelopus* species in the Costa Rican and the Panamanian tropical rainforest and how important it is to conserve these Central American native amphibian species that are threatened with extinction due to the chytrid fungus, *Batrachochytrium dendrobatidis, (Bd).* The Panamanian golden frog *(Atelopus zeteki)* one of the species severely affected by Bd, is the national symbol of luck in the Panamanian culture. As a Panamanian, I have always appreciated the flora and fauna biodiversity of my country. In addition, Bd has also been found in amphibians native to Washington State (Hayes et al., 2009). My thesis will benefit professionals and concerned citizens interested in aquatic habitat conservation. We must understand the role of ecological and bioclimatic factors that contribute to amphibian decline, and my positionality will maintain neutral to the interpretation of the results. This means that there is a real issue that is affecting amphibian species in the tropics and around the world, and we need to find ways to help protect and conserve these and other species affected by Bd.

In chapter 2, my literature review, I discuss the importance of amphibians species, the biology and ecology of *Batrachochytrium dendrobatidis* (Bd), theories of the origin of Bd, the harlequin frogs (*Atelopus* species) of Panama and Costa Rica, Bd prevalence throughout Panama, the relationship between Bd and climate change, the conservation challenges Panamanian amphibians face, amphibian survival, the effect of ecological variables in amphibian microbiome against Bd, and the reintroduction of the *Atelopus limosus* to the wild.

In chapter 3, I discuss the methods used to create forensic history of 8 *Atelopus* species in Panama and Costa Rica using literature dated from 1972 to 2019 to provide a timeline of Bd detection, *Atelopus* decline, extinction and recovery, and *Atelopus* life history. In my second methods, I discussed how DNA sequence data was collected to produce a phylogenetic tree and analyze the connection of the *Atelopus* species genetics with extinction or decline. In my last method, I discussed how I used R (R Core Team 2020) to produce a species distribution model using unbiased data to analyze climatically suitable habitats based on historical bioclimatic variables from 1970 to 2000.

In chapter 4, I discuss the results of the forensic history of 8 *Atelopus* species in Costa Rica and Panama, the *Atelopus* species phylogenetic tree and the connection with their extinction and decline and discuss the correlation between Bd prevalence and bioclimatic variables.

In chapter 5, the discussion, I review and verify the findings, and discuss the importance of bioclimatic variables and ecological traits, and genetic similarities that are connected with Bd prevalence and amphibian extinction. I also present the limitations of the thesis, next steps and future studies, and a conclusion of the thesis.

CHAPTER 2 Literature Review

2.1 Introduction

Amphibians are the most vulnerable animals in the world. According to the Amphibian Survival Alliance (2017), of the over 6,000 amphibian species in the world, 42% are threatened to become extinct (Amphibian Red List Authority, 2017). Since the 1980s, a total of 122 amphibian species are critically endangered, and of these species, 113 are extinct in the wild, most of them from Central and South America (Baillie et al., 2004). Additionally, 34 extinct amphibian species have been reported, of which 20 of these species, endemic from Sri Lanka, vanished more than one hundred years ago (2004). Moreover, extinctions may have accelerated in recent years. In the 1980's, 11 of the 34 species disappeared suddenly, including the Australian Gastric-brooding frogs, (*Rheobatrachus spp.*), the Costa Rican Monteverde Golden frog (*Incilius periglenes),* the Wyoming toad (*Bufo baxteri*), the Panamanian golden frog (*Atelopus zeteki*), and the Puerto Rican Golden Coqui frog (*Eleutherodactylus jasper*) (Berger et al., 1998). Most amphibian decline happened in tropical areas with elevation above 500 meters in Central America and above 1,000 meters in South America, where the majority of these endangered amphibians are endemic to their home range (Collins, Crump, & Lovejoy, 2009). Bd has affected amphibian species in mountainous areas.

Bd is threatening the Darwin's Frogs (*Rhinodermatidae*) from Chile and Argentina the New Zealand frogs (*Leiopelmatidae*), and another frog family (*Bufonidae*) including the Harlequin frog (*Atelopus varius*) from Panama and Costa Rica, is at risk to become extinct (2004) (Baillie et al., 2004). The chytrid fungus is causing a major impact on many amphibians on a global scale.

The decline of amphibians has been so overwhelming that about 40% of amphibians in Costa Rica were extinct by the late 1980s (Baillie et al., 2004). One major factor of the extinction of these species may have been the fungal disease, Chytridiomycosis caused by an amphibian fungal pathogen, *Batrachochytrium dendrobatidis* (Bd) (2004).

Two amphibian species, endemic to Panama and Costa Rica are likely extinct in the wild due to Bd: The Harlequin frog (*Atelopus limosus)* and the Panamanian Golden frog, (Lips et al., 2008), the national symbol of good luck in Panamanian culture. Other *Atelopus* species endemic to Panama and Costa Rica are experiencing severe population declines and are threatened with extinction.

The *Atelopus* species may be the most impacted in Central America. Rohr et al. (2008) suggest that sixty-seven species were extinct since the 1980s. The harlequin frog (*Atelopus varius*) have disappeared from its natural habitat in Costa Rica and Western Panama due to Bd, and possibly related to interactions with climate change. The spread of this fungal disease approached the East of Panama. (Crawford et al., 2010; Lips et al., 2008). Although a study revealed that Bd has spread far East of Panama in the region of Tortí (Rebollar et al., 2014), there has not been a study of Bd detection in *Atelopus* species in East Panama.

Many anthropogenic factors have contributed to the loss of amphibian biodiversity around the world (Young et al., 2001). One factor of amphibian extinction is introduced species, which can prey on or compete with native species (Young et al., 2001). Another factor is overexploitation; for example, international trading (Rosenblum et al., 2010). Factors like contamination with chemicals such as pesticides and fertilizers can kill amphibians (Collins et al., 2009). However, different patterns of temperature can also affect amphibian biodiversity (Young et al., 2001), and diseases like Chytridiomycosis caused by Bd leads to amphibian decline (Lips et al., 2008). One main reason of the amphibian extinction around the world may be the connection between Bd and climate change.

If Bd is one major factor of amphibian population decline and extinction, what makes it so prevalent? How can Bd impact the aquatic habitat in the tropical rainforest? What is the relationship between the amphibian chytrid fungal disease and climate change?

In this literature review, first I discuss amphibians from an ecological perspective and how we have benefited from them. Then I cover the biology, ecology, and pathology of *Batrachochytrium dendrobatidis* (Bd), the various origins of Bd, the history of Bd and amphibian extinction, the connection of Bd with climate change, how some amphibians may be recovering from Bd. I also discuss efforts to reintroduce *Atelopus limosus* to the wild.

2.2 The Importance of Amphibian Species

Why should we care about the loss of amphibians? Amphibians contribute to science and education as well as our self-benefit. We should care about amphibians as much as we should care about any other animal or plant species (Dodd Jr., 2009). We use amphibians as pets and food, and as test subjects for medical research, and schools (2009). Amphibians can contribute to finding cure for many human health problems. The loss of amphibian species biodiversity can create a disadvantage in scientific research for human health (Halliday, 2008). This means amphibians have been playing an important part in saving human lives and in our preparation to higher education.

Amphibians are an important part of nature as both prey and predator. They contribute to maintaining an ecological balance and are vital for the survival other living organisms in forest and wetland ecosystems in the Neotropics (Valencia-Aguilar, Cortés-Gómez, & Ruiz-Agudelo, 2013). This means that amphibians are important part of the food chain as food source to other animals.

Habitat alteration can impact amphibian biodiversity. Amphibians can negatively respond to ecological changes, for example, change in the environment and pollution, as they need water to survive (Halliday, 2008). Halliday indicated that amphibian's skin does not absorb water, so they depend on aquatic habitats (2008). As contributors to scientific research and human health, amphibians need to be protected around the world.

2.3 Life History and Transmission of Bd

Chytridiomycosis is a rare disease. Bd infects amphibian species (Longcore et al., 1999). One study found that Bd reproduces asexually, and it consists of two fundamental structures: "a round, permanent zoosporangium, about 10 to 40 micrometers (μm) in diameter, and a zoospore with moving flagellums about 2 μm in diameter, which mobilize quickly to find a substrate to settle" (Rosenblum et al., 2008). In this case, the substrate is the frog's skin. The reproductive body, zoosporangium, develops zoospores and then discharges them from its tubes into the surrounding environment (Longcore et al., 1999). Collins et al., (2009) indicated that zoospores are Bd's only reproductive stage and zoospores need water to discharge (2009). The zoospores attach to the host's skin and reproduce zoosporangia (Figs. 1 and 2, Longcore et al., 1999; Collins et al., 2009).

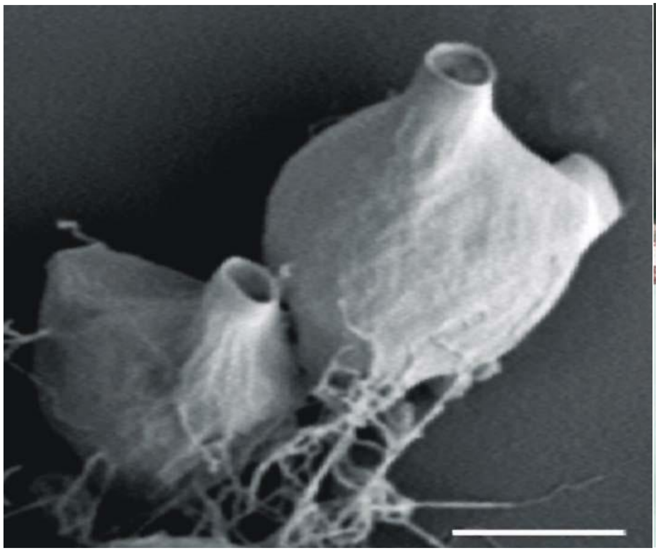


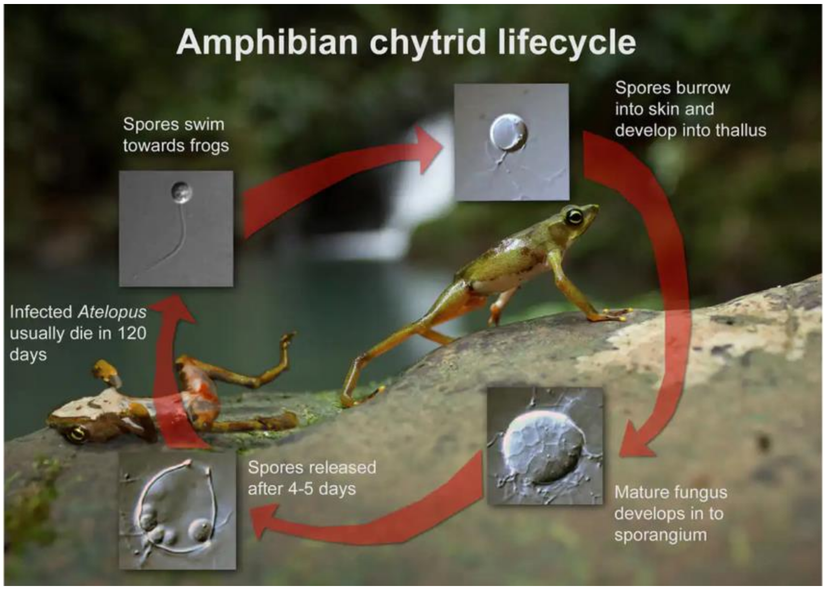
Figure 2. Zoosporangium releases zoospores from its tubes. Source: Longcore et al. (1999).

Figure 1. Redness of the skin indicates the presence of Bd. Bottom: Loss of beak and Bd infection on legs, face and upper body. Source: Longcore et al. (1999).

2.4 Pathology of Bd

What makes Bd so deadly to amphibians? One study revealed that Bd swims in the water, then enters the amphibian skin and zoospores colonize the skin cells, thickening the outer layer of the frog's skin (Rosenblum et al., 2010). Because amphibians use their skin as a respiratory function, the infected frogs suffocate and die (Rosenblum et al., 2010). Once the frog is dead, Bd comes out of the skin of the dead frog and goes back into the water to find a new host (Fig. 3, Rosenblum et al., 2010). Kirshtein et al. (2007) indicate that Bd thrives in water. Walker et al., (2007) analyzed water and sediment samples from ponds in Spain and found that Bd can live 12 weeks in moist riverbanks and rocks without an amphibian host. These findings may explain the reason Bd is very persistent in aquatic environment.

Figure 3. Bd lifecycle starts when spores burrow into the frog's skin. Source: Rosenblum et al. (2010).

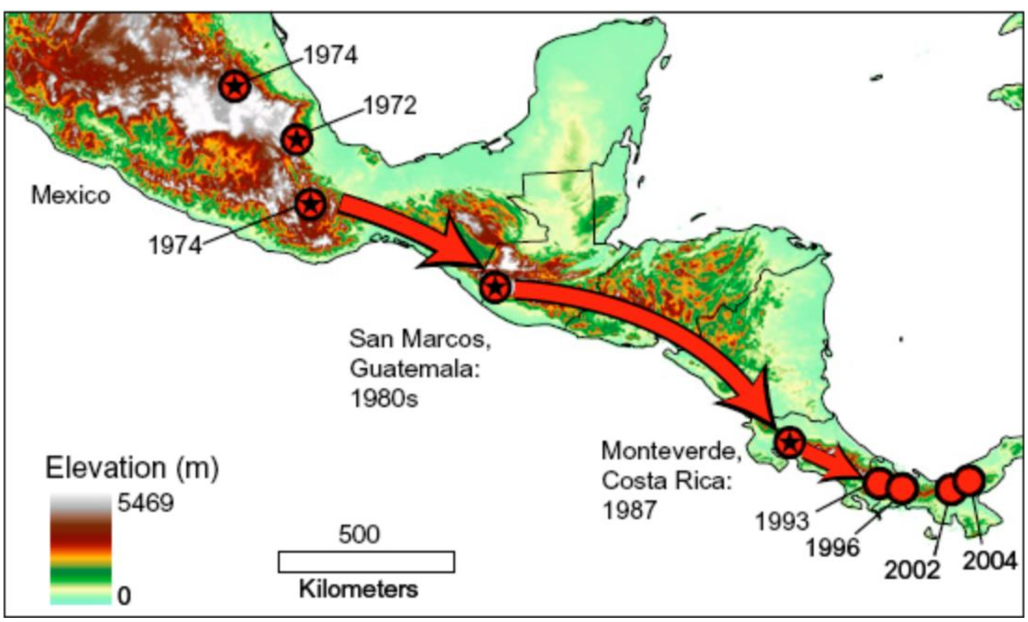


The infection process of Bd takes place in water bodies, often in mountainous areas. A research study found that there are three ecological characteristics of Bd, (Baillie et al., 2004). Bd thrives in cooler temperature, but cannot survive warmer temperatures, which means that amphibians living in higher elevation will be affected by Bd than the lowland amphibians. Bd only thrives in freshwater habitats, which explains the decrease of amphibians living near streams (Baillie et al., 2004). Bd is more common in forests with humid climate and is likely to infect amphibians breeding upstream.

Bd may be the cause of the loss of amphibian biodiversity in Central America. How does Bd affect amphibians? Bd affects the frog's skin layers and the nervous system (Longcore et al., 1999). For example, a sick frog may have faded skin, rough, peeled skin layer, especially on their feet (Frog Chytrid Fungus, 2017). Affected frogs' legs are spread away from its body, instead of tucked in, and in worst cases, the frog's back legs lose movement and drag behind it (Frog Chytrid Fungus, 2017). Tadpoles can also become infected. Bd infects the beak when the tadpole is in a metamorphosis stage. (Longcore et al., 1999). As the tadpole develops, Bd will spread in the legs, and as it grows into a frog, the Bd will spread in any part of the skin (Longcore et al., 1999). Longcore et al. (1999) have found that frogs and tadpoles can get infected with Bd by direct contact between them and through exposure to infected water.

Bd is presumed to be the main factor of amphibian fluctuation in the neotropics. Neotropics are regions from Central and South America, southern Mexico and the Caribbean. Although the amphibian declines have been reported since 1970 (Fig. 4, Cheng et al. 2011) and has been affecting the endemic species from the border of Mexico to the tip of South America, Bd was first identified in 1998 as the cause of amphibian decline in North and Central America, Europe, and Australia (Crawford et al., 2010; Kilpatrick et al., 2010). The loss of endemic amphibian species may have a significant ecological impact on the aquatic habitat.

Figure 4. Map of Bd detection from Mexico through Central America. Source: Cheng et al. (2011).



2.5 Origin of *Batrachochytrium dendrobatidis* (Bd) and the Cause of its Spread

Two studies have been conducted to find the first incident of the deadly infectious disease. The two main theories are that the origin of Bd is either African (Weldon et al., 2004 used samples of *Xenopus spp.*, which are native to Africa), or Asian (O'Hanlon et al., 2018 used amphibian genomes to find when Bd began to spread for the first time). Although both theories may have compelling evidence, there are still many challenges in finding the actual timing of the origin of Bd.

Weldon et al., (2004) state that Bd could have originated in Africa. They studied 697 specimens of 3 *Xenopus* species collected between 1879 and 1999 (Weldon et al. 2004). The study showed that Chytridiomycosis was present in African clawed frogs (*Xenopus laevis*) specimens dated in 1938, (Weldon et al., 2004). The researchers indicated that Bd was found in these African endemic species more than 20 years before reports of the first case of Bd beyond this continent (2004). This theory is suggesting that Bd may have originated in Africa and spread around the world.

The other theory (O'Hanlon et al., 2018) argues that Bd could have originated from another part of the world. O'Hanlon et al. conducted a study using genetic lineage to identify the source of Bd on infected amphibian populations from Africa, North America, South America, East Asia and Japan (2018). O'Hanlon et al. collected 177 samples of genomes to arrange in sequence and incorporated analysis of sample genomes from Ferrer et al., (2011), Rosenblum et al. (2010), and Weldon et al., (2004). A total of 234 samples, of which were isolated for study (2018). The analysis found lineages from Africa, Europe, Brazil, and one global lineage (O'Hanlon et al., 2018). Lineages found in the European samples which the researchers thought came from Switzerland have now been classified in the Asian lineage because of another ancient Asian lineage that was found in the North American bullfrogs with a genetic relation to an endemic amphibian species in Brazil (O'Hanlon et al., 2018). These results indicated that multiple Bd lineages were found in endemic amphibian species in Asia and then globally spread in East Asia, during the early 20th century when the global amphibian trade increased. O'Hanlon et al. (2018) used phylogenetic analysis to trace the source of Bd in Asia.

These two theories bring persuasive argument about the origin of Bd. It may be safe to say that DNA sequencing of Bd from around the world may be an accurate analysis. However, there are still many challenges in finding the actual timing of the origin of Bd.

2.6 How did Bd Spread Around the World?

The international trade of one frog species may have caused the spread of the chytrid fungus. Bd spread started with the exportation of African clawed frogs (*Xenopus laevis*) used for human pregnancy testing in the 1940s and 1950s (Weldon et al. 2004). The pregnancy test functioned when the urine of pregnant women was injected into the African clawed frog triggering its ovulation due to the high levels of estrogen in the urine (Weldon et al., 2004). Rosenblum et al. (2010), suggested that international trading of the American bullfrog (*Rana catesbeiana*) may have caused the spread of Bd in other countries, as Bd was first found in this amphibian species in South Carolina in 1978. The international trade of the African clawed frog may have introduced Bd around the world including the United States.

2.7 Major Trends in Amphibian Extinctions

To understand the gravity of the problem, the International Union for Conservation of Nature (IUCN) categorized all known amphibian species by their conservation status through a global species assessment (Baillie et al., 2004). They found that amphibian diversity is declining around the world. The IUCN assessed a total of 6,892 amphibian species, of which 6,098 species are frogs and toads. 40% (2,409 species) are extinct or threatened (Table 1, IUCN, 2020). 80% of *Atelopus* species are critically endangered and 70% have declining populations (IUCN, 2020). About 30 out of the 113 *Atelopus* species are extinct in the wild (La Marca et al., 2005). Bd has caused the population decline and loss of the Harlequin frog *(Atelopus varius)* across Central America (La Marca et al., 2005; Lips et al., 2008). The loss of 3 *Atelopus* species in Costa Rica and Panama could have a negative effect in their ecosystems.

Table 1. IUCN 2019 assessment of the amphibian species by groups. From the 6,892 of amphibians assessed 6,098 species are frogs and toads, and 40% (2,409 species) are extinct or threatened.

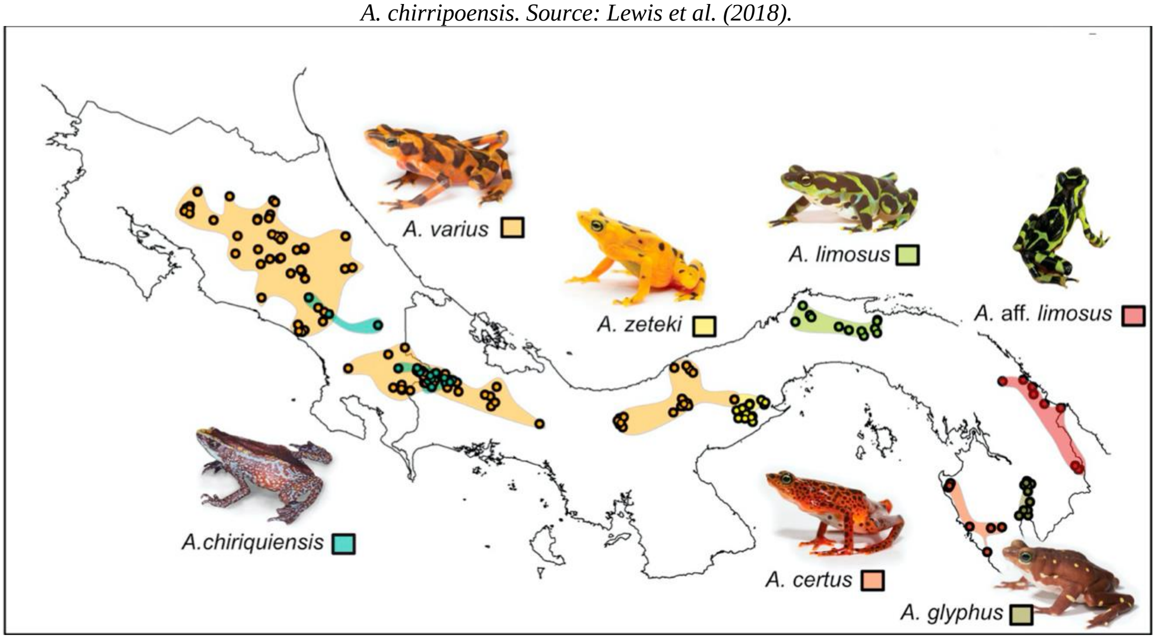
ORDER	EX	EW	CR/ PE	CR PEW	CR	EN	VU	NT	DD	LC	TOTAL	% Threatened/ extinct
Anura Frogs & Toads	32	2	124	3	502	851	565	330	1192	2624	6098	40%
Caudata Salamanders & Newts	3	0	7	0	107	133	104	55	42	167	611	67%
Gymnophiona Caecilians	0	0	0	0	1	9	4	2	102	65	183	9%
Subtotal	35	2	131	3	610	993	673	387	1336	2856	6892	41%

2.8 The Harlequin Frogs (Genus *Atelopus*) of Panama and Costa Rica

Located in Central America, Costa Rica, and Panama tropical rainforests are the richest and the most biodiverse regions in the world (Stephen Fry, 2015). Historically, these two regions had the most amphibian abundance than Borneo and the Philippines (Norman J. Scott, Jr., 1976). However, these regions suffered a decline in their amphibian populations. For example, Harlequin frogs, genus *Atelopus*, once abundant, are distributed from Costa Rica down to Bolivia and in the Southeast or the Guianas (González-Maya, Gómez-Hoyos, Cruz-Lizano, & Schipper, 2018), have significantly declined since the late 1980s. There are 113 *Atelopus* species dispersed from Costa Rica to South America, 42 species have decreased 50% of their population from 1984 to 1996, and only 10 are stable (LaMarca et al. 2005). Costa Rica and Panama harbor 362 amphibian species (Dirzo and Bonilla, 2013), but there are only 8 documented *Atelopus* species distributed through Panama to Costa Rica (Fig. 5) (Savage, 1972). Panama has six described species of *Atelopus*: *A. certus*, *A. chiriquiensis*, *A. glyphus*, *A. limosus*, *A. varius*, *A. zeteki*, and at least one undescribed species: *Atelopus* aff. *limosus* (Flechas et al., 2017). The only Panamanian *Atelopus* species that occupy one region close to the Costa Rica-Panama border are the *A. chiriquiensis* and the *A. varius* (Savage, 1972). The *Atelopus senex* is a described species from Costa Rica and the *Atelopus chirripoensis* is possibly extinct in Costa Rica (Savage and Bolaños, 2009).

This *Atelopus* genus is dangerously approaching extinction and is perhaps the most threatened in the world (Lewis et al., 2019). The tropical rainforests of Central may be losing their biodiversity and can expose a change in their ecosystems. Costa Rica suffered the decline of the Monteverde golden toad (Pounds & Crump, 1994) and the decline of the *Atelopus chiriquiensis* due to Chytridiomycosis (Lips, 1998). The loss of the *Atelopus* species can negatively impact the tropical rainforest ecosystems in these regions.

Figure 5. Distribution map for Atelopus species in Costa Rica and Panama. Species missing on this map: A. senex and A. chirripoensis. Source: Lewis et al. (2018).



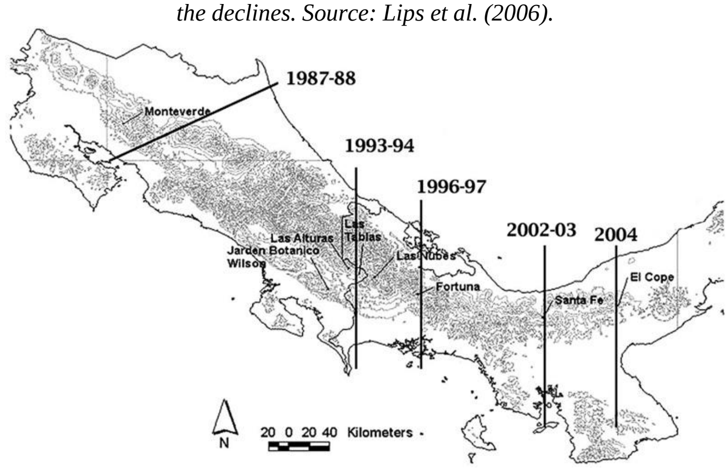
Abiotic factors, such as temperature, precipitation, and climate, can be influenced by the altitude of a region. Cold tolerance is frequently intense at high altitudes, and species of the same genus living in the same habitat demonstrate significant differences (Vo and Griddi-Papp, 2017). Most *Atelopus* species are endemic to remote tropical areas in high elevation. All species living in elevations above 1000 m have declined, and 75% have disappeared (LaMarca et al., 2005). High elevation, and precipitation and cool weather make an ideal condition for Bd to spread. There were no data collected before the Bd epidemic started in Central Panama (Crawford et al., 2010). The endemic amphibians may not be able to survive if Bd is prevalent in their home range. The remaining 25% of the *Atelopus* population may become extinct if Panamanian and Costa Rican authorities do not have a mitigation plan to conserve these species.

The disappearance of endemic amphibian species in Costa Rica and Panama is staggering. The golden toad *(Incilius periglenes)* and the harlequin frog *(Atelopus varius)* were abundant in Monte Verde, Costa Rica from 1972 through the late 1980s (Pounds & Crump, 1994). However, after 1988 both species started to decline in population and by 1992, both species have vanished (1994). The Panamanian Golden frog population was abundant in El Valle de Antón in Central Panama about 15 years ago, but by 2004 the frogs vanished (Kolbert, 2014). The amphibian population is being affected by an infectious skin disease caused by Bd (Rodríguez-Brenes et al., 2016), and has spread across the Eastern side of the Panama Canal.

2.9 Detailed history of Bd and Amphibian Extinction in Panama

In 1998, scientists found that the amphibian population in the mountains of Panama is decreasing. Dr. Crawford, Dr. Lips, and Dr. Bermingham, (2010) from the Smithsonian Tropical Research Institute (STRI), surveyed the loss of amphibian species diversity in the tropical rainforest of Central Panama, located in El Copé, in the province of Coclé, after the detection of Bd in Costa Rica in the 1980s (2010). In 1998, Crawford et al. (2010) conducted a study of Bd spread at Omar Torrijos National Park, with an elevation of 800 m, another location near El Copé. Crawford et al. (2010) indicated that there is no data collected before the Bd epidemic started at El Copé in 2004. All these locations are on the west side of the Panama Canal (Fig. 6).

Figure 6. Map of Costa Rica and west Panama with sites of amphibian declines. The lines mean date and location of the declines. Source: Lips et al. (2006).



The scientists identified the amphibian species most affected by Bd. Crawford et al. (2010) first detected Bd in 2004 in El Copé, then collected data on the number of amphibians and conducted a 7-year comparison of their data of diversity loss caused by Bd. Using a DNA barcode method to identify the diversification and lineage of amphibian species, Crawford et al. (2010) found 63 Panamanian amphibian species of which are categorized by taxonomic families including *Aromobatidae, Bufonidae; Caecilidae* (a caecilian) and *Plethodontidae (*salamanders) among others, before the amphibian decline caused by Bd. An additional survey was conducted between 2006 and 2008 and found that out of the 63 known amphibian species, 25 were extinct (2010). A genetic analysis was conducted out of the remaining 11 known amphibian species in the Panamanian tropical rainforest and found that 5 species lineages are extinct with a total loss of 30 amphibian species caused by Bd (Table 2, Crawford et al., 2010). The amphibian chytrid fungus could be slowly spreading across the eastern highlands of the Panamanian tropical rainforest, and the decline of amphibian species is alarming.

Table 2. Amphibian decline in El Copé, Central Panama. Loss of diversity was measured by four indicators: named species, candidate species, lineages (named + candidate species), and PD (sum of branch lengths obtained by MPL analysis of phylogenetic tree). Source: Crawford et al. (2010).

Species removed (by decline category)	Named Species lost (n = 63)	Candidate species lost (n = 11)	Lineages lost (n = 74)	PD lost (%)
Extirpated and DD-extirpated	25 (40%)	5 (45%)	30 (41%)	33
Extirpated, DD-extirpated and critical	34 (54%)	5 (45%)	39 (53%)	41
Extirpated, DD-extirpated, critical and declined	42 (67%)	6 (55%)	48 (65%)	61

2.10 Bd Spread Across the East of the Panama Canal

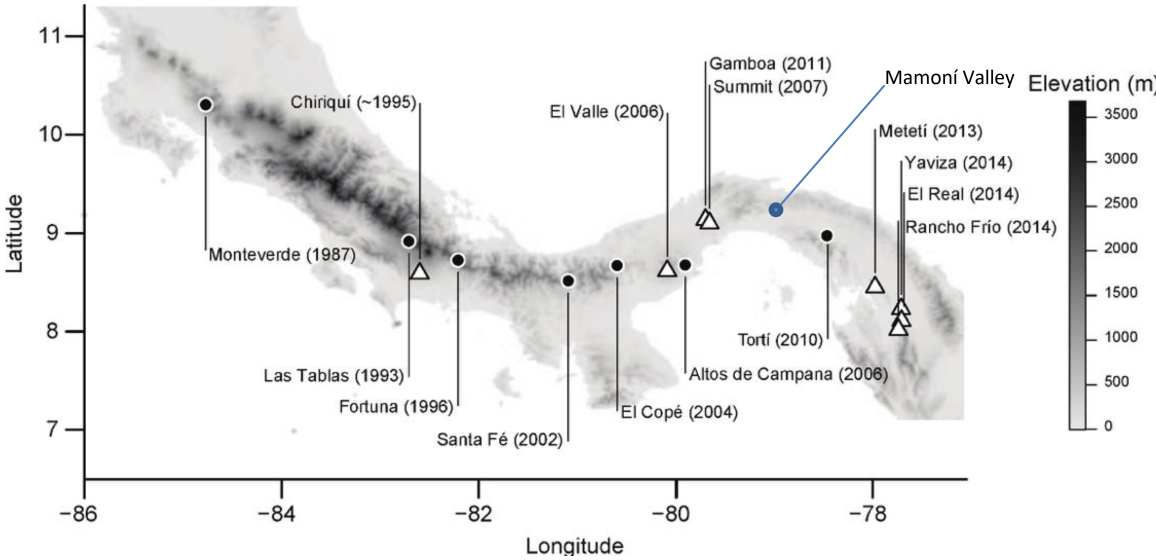
Bd is presumed to be spreading in lowlands in Panama. Not only can Bd spread in streams of mountainous areas, but also the lower elevations of the Panamanian tropical rainforest. Rodríguez-Brenes et al. (2016), focused their study on the spread of Bd in the Túngara frogs (*Engystomops pustulosus*) that inhabit the lowlands of Panama (2016).

Ibañes et al., (1999) describe the Túngara frog as a small frog with a squat body and short limbs with a grayish-brown dorsal surface. Males are about 33 mm long, and females are about 36 mm long (1999). Ibañes et al. indicated that Túngaras are also found in streams up to 1,500 m in elevation and can live in different types of habitats (1999). However, Túngaras are most likely to thrive in warmer environments. Vo and Gridi-Papp (2017), argue that Túngara frogs live in tropical freshwater habitats with warm temperatures and do not respond well to cold temperatures (2017).

Bd was spreading more slowly on Túngara frogs than any other amphibians in the lowlands of Panama. Vo and Gridi-Papp measured the rate of Bd spread in the Túngaras since the first case of Bd positive in Túngaras and concluded that the Bd is spreading at a slower rate in Túngaras in the lowlands at 34 miles/year, comparing to rate spread in other amphibians in the same location at 19 to 108 miles/year (2017). Vo and Gridi-Papp found that the rate of Bd spread from three locations along the Panama Canal watershed, Summit, Chagres and Gamboa, were the slowest calculating it at 5 miles/year (2017). In addition, amphibian species most vulnerable to Bd infection are populations that occur in higher elevation (Woodhams et al., 2008). This means that the low rate of Bd spread may be affected by the lowland habitats with warmer temperature. This is important because amphibians in the lowlands may be able to survive Bd.

Bd spread slowly approached the Eastern side of Panama (Fig. 7). Rodríguez-Brenes et al., (2016) analyzed the Túngara frogs from 2009 to 2014 during June and November in 7 sites (2016). The sites were Chiriquí in the far west, El Valle in Central Panama, Gamboa and Summit, located near the Panama Canal, Metetí and Yaviza in the East, and El Real and Rancho Frío located in the in the Darien National Park, in the far East of Panama (Rodríguez-Brenes et al., 2016). Rodríguez-Brenes et al. concluded that the Western and Central sites were Bd positive as predicted (2016). Although the Gamboa area was Bd negative in 2010, one year later in 2011, Bd was detected in Túngaras, and by 2014, all sites were tested positive for Bd (Rodríguez-Brenes et al., 2016). Bd has extended throughout the highlands and the lowlands of Panama suggesting that more studies are needed to identify the cause of such change.

Figure 7. Timeline of Bd Detection Throughout Costa Rica and Panama



● Sites of Bd detection throughout Panama.
△ Sites of Túngara frog samples.
● Mamoní Valley was added to show the location of the released Limosa Harlequin frogs by STRI.
Source: Rodríguez-Brenes et al. (2016).

2.11 Is there a Relationship between Bd and Climate Change?

Early studies suggested climate change played a big role in extinction. Two studies were conducted to find the connection between Bd and climate change. Pounds et al., (1994) theorized that the Golden toads (*Incilius periglenes*) and Harlequin frogs *(Atelopus varius)* might have disappeared from the Costa Rican rainforest due to climate change (1994). Pounds et al. suggested that 20 anuran species were extinct in 4 years and other species declined but were not extinct (1994). To analyze their theory, Pounds et al. indicated that by 1992 the *Atelopus varius* became extinct in the Costa Rican Monte Verde tropical rainforest (1994). The authors analyzed patterns of precipitation, stream flow, weather, and seasonal El Niño patterns from July 1986 to June 1987 to examine changes in amphibian abundance may be related to climate change (1994). Pounds et al. indicated that the 1986-1987 cycle was the driest (1994). Wind flow has moved upward to the Caribbean slope, reducing moisture in the rainforest affecting amphibians (1994). The authors indicated that the decrease in rainfall between May 1986 and August 1987 and a decrease of stream flow between during July 1986-June 1987 cycle, as well as an increase of minimum and maximum temperatures (1994). The researchers found that some natural patterns coincide with their theory. Amphibian population decreased during El Niño season when precipitation was the lowest in Monte Verde, Costa Rica from 1986 to 1987 (Pounds et al., 1994). Pounds et al. concluded that the decline of amphibian biodiversity has a connection with climate change (1994). The authors indicated that climate change will impact higher elevations forests, (Pounds et al., 1994). This theory suggests that some amphibian species like the golden toad and the Harlequin frog may have been affected significantly by the dry conditions caused by El Niño season in 1987 and may have cause these amphibians to become extinct in Monte Verde. This is important because we may not know for certain the cause of their extinction.

Later, authors called this climate change theory into question. Blaustein et al. (2011) suggested that the temperature pattern hypothesis of Pounds et al. (1994) that cloud cover in higher elevations of the tropical rainforest can influence Bd spread may be questionable (2011). Blaustein et al. argues that Pounds et al. only analyzed the changes in temperature in higher and lower elevations respectively during the rainy season, when amphibians mate, and not during the dry season (2011). This means that Pounds et al. hypothesis may be insufficient to prove that Bd is influenced by climate change. This is important because all types of climatic factors must be considered to determine the cause of Bd in the tropical rainforest of Central America.

Another author, Lips (2008), called this climate change theory into question. Ecological conditions may have contributed to the spread of Bd. Lips et al., (2008), argued that environmental causes may influence the spread of Bd, not climate change (2008). Lips et al. (2008) researched the connection of Bd outbreak and climate change related to spatiotemporal patterns. The authors studied the data of the *Atelopus* species in conjunction with data of other amphibian species from Central America and South America to determine if Bd is invasive (Lips et al. 2008). They hypothesized that if Bd is a widespread infection, patterns that represent space and time would suggest that Bd spreads throughout endemic habitats. The authors also suggested that if Bd is indeed invasive, then Australia, Central, and South America should have the same space and time patterns (Lips et al. 2008). The authors used data from the last *Atelopus* alive found in the wild including classifications of habitat loss, introduced species, the cause of decline among others (Lips et al., 2008). The researchers compared another data from 2004 publication from the Research and Analysis Network for Neotropical Amphibians (RANA) containing the last dates known of amphibian species observed in the wild and used it as a substitution for the date of species extinct in the wild, although it does not contain the actual date of amphibian extinctions (Lips et al., 2008). The authors suggested that to better analyze the space-time patterns of Bd exposure at a location, they needed to use the proxy for the actual date of amphibian extinction (Lips et al. 2008).

Limited information of the first amphibian decline by Bd challenged the authors' research. Lips et al. (2008) indicated that their research would not determine the exact timing of Bd spread. The authors used spatial patterns to determine the amphibian decline by Bd. Lips et al. (2008) analyzed how error sampling from the last year amphibians observed in the wild (LYO) would affect the connection of amphibian decline with temperature in the from 1970 to 1998. They found in their analyses that Bd is most likely an introduced pathogen (2008). The authors indicated that local ecological changes can affect the population growth and spread of Bd and host vulnerability, consequently influencing amphibian abundance (Lips et al., 2008). Because of strong evidence that Bd thrives in cooler temperatures, the hypothesis of Pounds et al., (1994) is not well supported, (Baillie et al., 2004; Lips et al. 2008). Their theory (Lips et al., 2008) suggests that climate change may not be related to Bd spread in Central America. These findings bring another question: how these ecological changes affect amphibians and how are they connected to Bd spread?

The theories from Pounds et al. (1994) and Lips et al. (2008) made compelling arguments about whether or not climate change played a role in amphibian extinctions. Whether we believe these theories, amphibians are becoming extinct in the Costa Rican and the Panamanian tropical rainforest. We must consider that ecological changes may also be impacted by climate change.

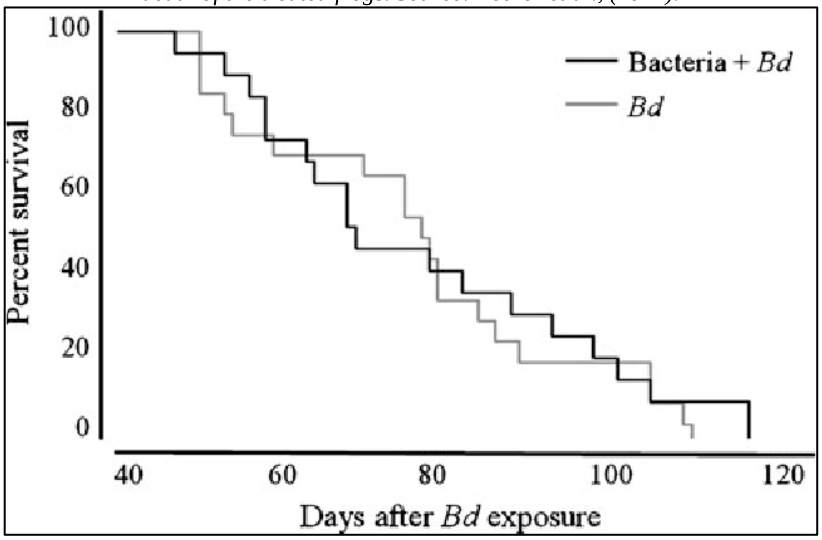
2.12 Panamanian Frogs Face Conservation Challenges

Endemic *Atelopus* species in the Panamanian tropical rainforest, especially the Panamanian Golden frog and the Harlequin frog, have been experiencing a population decline since the arrival of Bd. Efforts to increase *Atelopus* populations are underway. Species survival program in zoos across the United States have had successful results in mitigating the spread of Bd, on the Panamanian golden frogs (*Atelopus zeteki*) (Becker et al., 2011). Captive breeding can be the only method to ensure the survival of other

Atelopus species in Panama, like the Harlequin frog (*Atelopus certus*), the Clown frog (*Atelopus varius*), the Limosa harlequin frog (*Atelopus limosus*), and the Pirri harlequin frog (*Atelopus glyphus)* (Becker et al., 2011).

Becker et al. (2011) conducted a study to eradicate Bd using an antifungal microorganism. They experimented with 54 Panamanian golden frogs from the Houston Zoo with the purpose of mitigating Bd. The researchers used skin bacteria called *Janthinobacterium lividum* that was known to help mitigate the Chytridiomycosis in amphibians in the United States, on 1 group of Golden frogs to compare it with a control group infected with Bd (Becker et al. 2011). The study lasted about 120 days due to the overwhelming spread of Bd in both the control group and the treated group (Fig. 8), and the *Janthinobacterium lividum* bacteria was not present after the death of the treated frogs (Becker et al. 2011). The numbers of dead frogs in both groups were the same after 50 days, and one factor that negatively affected the frogs could have been the captive environment the frogs were living before the experiment. (Becker et al., 2011). There were successful studies in the United States, perhaps due to the difference of climate the treatments were conducted. Many Panamanian frog species will stay in captivity to conserve their species until scientists find a way to mitigate this disease.

Figure 8. Survival patterns of frogs treated with J lividum before Bd exposure and frogs exposed to Bd without treatment. 118 days after exposure the treated frogs were infected by Bd. J lividum bacteria was not present at the death of the treated frogs. Source: Becker et al., (2011).



Another study has been conducted to determine if one *Atelopus* species was ready to return to its environment. In efforts to reintroduce the captive Panamanian Golden frog to the wild, DiRenzo et al. (2014) tested the intensity of Bd in 5 *A. zeteki* that were never exposed to Bd and 3 *Atelopus zeteki* injected with to Bd strain JEL427-P39 23 weeks before the experiment. DiRenzo et al. (2014) analyzed the quantity of zoospores in the frogs' bodies at the time of death (Table 3). When the experiment started, the authors treated each frog with 30,000 Bd zoospores for 10 hours (2014). 7 days after the inoculation, all frogs were monitored each day for any symptoms and tested for Bd intensity. The days of survival for the naïve group was between 18 – 31 days with variable Bd zoospore intensity. The days of survival from the previously exposed group were between 18 and 33 days with variable Bd zoospore intensity. The last frog from the previously exposed to Bd group survived for 33 days with no Bd zoospores by the time of death (2014). The experiment from DiRenzo et al. indicated that the Bd intensity in the *A. zeteki* at time of death was very high in comparison to other studies (2014). The authors suggested that the *A. zeteki* is extremely vulnerable to Bd infection concluding that these amphibians are not capable to being reintroduced to the wild.

Table 3. Atelopus zeteki infection intensity (number of zoospores on skin swabs) and zoospore output (number of zoospores released per minute) at death. Source: DiRenzo et al. (2014).

Prior exposure	Total days survived post-inoculation	Bd infection intensity at death	Zoospore output at death
Naïve	21	520,436	3.5
Naïve	28	1,697,306	0.0
Naïve	18	4,454,759	4.9
Naïve	31	8,781,016	0.2
Naïve	25	9,584,158	170.6
Previous	18	2,291,631	7.1
Previous	33	2,960,916	0.0
Previous	31	4,385,154	0.0

2.13 Are Amphibians in Panama Surviving Bd?

After the Bd outbreak in 2004, scientists believe that some amphibians are recovering from Bd in the Panamanian tropical rainforest. In 2017, an *Atelopus* species was observed at the same Bd prevalent site in El Copé, in Central Panama, after the Bd wave of 2004 (Voyles et al., 2018). Voyles et al. (2018) conducted an analysis on the frog behavior and resistance to observe changes in Bd spread in the Harlequin frog (*Atelopus varius)* and the Australian green tree frog (*Litoria caerulea)* species. The researchers used Bd zoospore samples collected in 2004 (historic) and Bd zoospore samples collected in 2013 (current) from sites in Panama (Voyles et al., 2018) to compare the Bd intensity in the frogs. Voyles et al. (2018) indicated that there was no difference in the intensity of Bd between the two frog species.

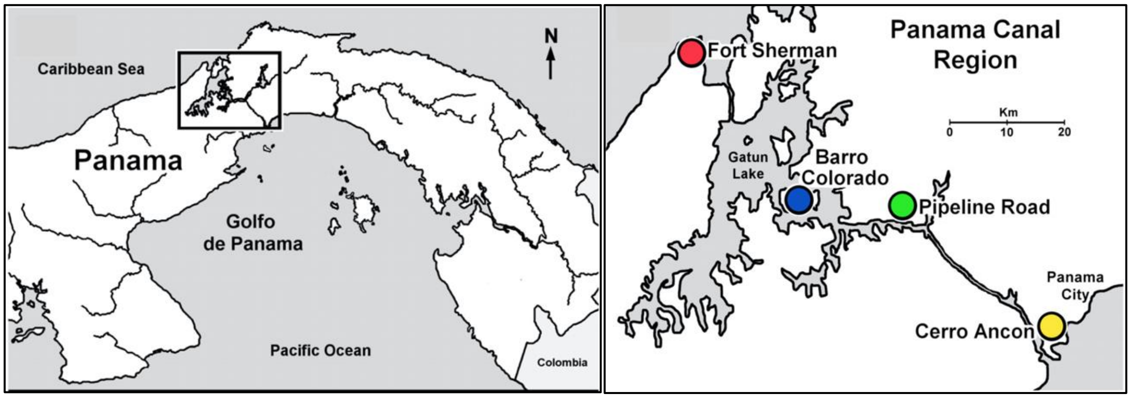
The authors also tested both historic and current Bd samples to observe any difference in Bd zoospore growth rate (Voyles et al., 2018). Voyles et al. (2018) suggested that there was no difference of Bd intensity in both *Atelopus varius* and *Litoria caerulea*. The authors looked close at the phylogenetic construction from the historic and current Bd samples and found no difference or any change in the Bd infection.

Finally, the researchers tested skin secretions samples from 6 different frog species taken before and during the outbreak, and from captive and wild *Atelopus varius* species from 3 different Panamanian sites to determine the resistance against Bd (Voyles et al., 2018). They found differences of skin secretions efficiency among the amphibian from the two time periods. The wild *Atelopus varius* skin defenses were more capable to resist Bd than the captive *Atelopus varius* species, concluding that some amphibians have developed stronger skin defenses. Panamanian amphibian species are showing signs of recovery from Bd in 3 sites near El Copé, resulting from their skin secretions response to resist Bd throughout the years (Voyles et al., 2018). There may be an opportunity for study to determine if the skin secretion from the *Atelopus varius* can be successfully used in other *Atelopus* species to help resist Bd.

2.14 The Effect of Ecological Variables in Amphibians' Skin Defenses Against Bd

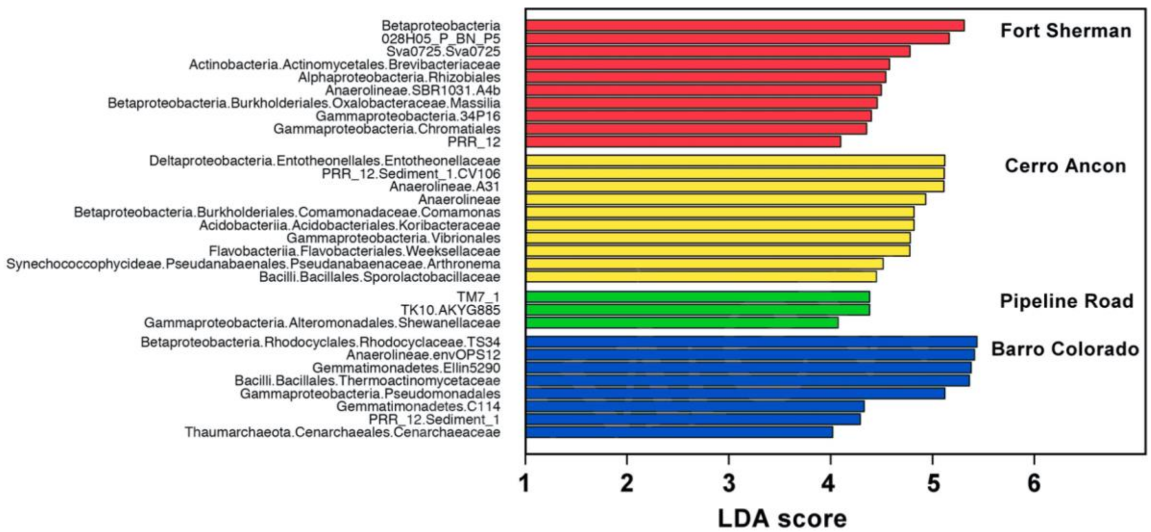
Ecological factors (precipitation, soil pH level) play a role in how the frogs' skin bacteria affect survival against Bd. Varela et al. (2018) studied the variation of the amphibian skin microbiota of three frog species: 58 green and black poison frogs (*Dendrobates auratus)*, 6 rainforest rocket frogs (*Silverstoneia flotator)*, and 6 Talamanca rocket frogs (*Allobates talamancae).* They compared 4 sites across the Panama Canal that have different soil pH and precipitation levels: Cerro Ancón, an urban forest with an elevation of 564 ft; Ft. Sherman (171 ft above sea level) located on the Panama Canal watershed in the Atlantic Ocean, Pipeline Road in Gamboa (167 ft. high) located along the Panama Canal watershed, and Barro Colorado Island (394 ft. high) that sits in the middle of the Panama Canal. These sites are considered old growth forests (Fig. 9, Varela et al. 2018).

Figure 9. Map of Panama and the four sites studied. Source: Varela et al. (2018).



No frog in this study tested positive for Bd, but Bd is prevalent near the Panama Canal (Varela et al. 2018). The effect of the soil pH on the abundance of the skin bacteria was examined before and after the rainy season. As a result, the variation of skin bacteria in all 3 species are different by soil pH and rainfall. The skin betaproteobacterium *Janthinobacterium lividum*, that produces compounds that prevents Bd growth (Brucker et al., 2008), increased in 2 amphibian species at the beginning of wet season. However, their skin bacteria diversity decreased during dry season and low soil pH levels, and precipitation had no effect on the skin bacterial diversity (Varela et al., 2018). They compared bacterial richness (alpha) and the changes in diversity (beta) in each frog species. In the *D. auratus*, bacterial diversity had a negative correlation with the soil pH level (Varela et al., 2018). Fort Sherman had the highest bacterial diversity and the lowest soil pH level, Cerro Ancón and Pipeline Road in Gamboa were in the middle, and Barro Colorado had the lowest bacterial diversity and the highest soil pH level (Varela et al., 2018). The authors found that in Pipeline Road, the *D. auratus* had the lowest skin bacterial diversity than the *A. talamancae* and the *S. flotator* suggesting that the *A. talamancae* is more vulnerable to Bd than the other two species (Fig. 10). Amphibian skin bacteria diversity and abundance play an important role in inhibiting Bd growth. Although there is a significant variation of bacteria among these studied amphibians, changes in the environment can affect amphibian microbiome structure.

Figure 10. Bacterial community composition in D. auratus across the sites near the Panama Canal. Varela et al. (2018) used linear discriminant analysis (LDA) scores to find the most significance in bacterial composition. Source: Varela et al. (2018).



2.15 Reintroduction of the *Atelopus limosus* into the Wild

The East of Panama has yet to be hit with Bd spread and could be an ideal environment for experimenting with the survival of one *Atelopus* species in the wild. With the help of the Amphibian Rescue and Conservation Project in Panama, 90 Limosa harlequin frogs (*Atelopus limosus*) were released into the wild, in the Mamoní Valley (Eastern Panama), for the first time in May 2017 (First Release Trial, 2017). The purpose of this trial was to learn how these amphibians can survive in a natural environment after being in captivity (First Release Trial, 2017). Therefore, the success of the survival of the Harlequin frog in the wild may indicate an ideal habitat condition and a possible reintroduction of the Panamanian Golden frog.

The Limosa harlequin frogs *(Atelopus limosus)* chosen for reintroduction have never lived in the wild, so there is a probability that some frogs could die early in their cycle. Due to the uncertainty about the frogs' survival, the frogs were tagged with individual numbers and marked 30 frogs' toes with a rubber that glows with UV light for better identification (First Release Trial, 2017).

STRI started to slowly release the frogs by allowing them to stay in cages for 1 month in the wild, so that they could adapt to their new environment (First Release Trial, 2017). STRI scientists attached mini transmitters to monitor 8 of the 30 frogs and introduced another group of 8 frogs to the wild with no adaptation period as a control group (First Release Trial, 2017).

This project is still in progress. Dr. Roberto Ibañez from STRI indicated that the data on the reintroduction trial is being analyzed for publication (D. E. Lloyd personal communication, January 10, 2019).

2.16 Conclusion

The decline of the amphibian population caused by Bd is devastating. The sudden disappearance of the Gastric Brooding frog (*Rheobatrachus silus*) and the Southern Day Frog (*Taudactylus diurnus*) (Baillie et al., 2004) indicated that Bd has been prevalent in endemic amphibian species. According to Baillie et al., most of the amphibian declines have taken place in Central and South America (2004).

The endemic amphibians, including the Panamanian Golden frog, are rescued and kept in captivity. This species should not be reintroduced to the wild until we see positive results in the reintroduction of the Harlequin frogs. Efforts to conserve these beautiful species by STRI researchers and reintroducing some other amphibian species into the wild is very significant. However, this is only the beginning, and the survival of these amphibians is uncertain. Scientists are working together to find an anti-fungus to stop the mass eradication of the amphibian species around the world (Becker et al., 2011). The conservation of the amphibian species is vital to humans and other animal species, as they are essential in scientific research (Halliday, 2008).

While Bd affects amphibians, we do not know how Bd interacts with climate. The Bd fungus is mostly found in cool aquatic freshwater habitat, and its proliferation is most likely during the rainy season when the amphibians are in their mating stage (Rodríguez-Brenes et al., 2016). Bd has been shown to be a significant factor in the amphibian decline. However, the hypotheses of Pounds et al. (1994) and Lips et al. (2008) have yet to resolve with certainty how Bd caused amphibian decline relates to climate change.

Bd is still prevalent in the Costa Rican and Panamanian rainforest. Scientists have studied the degree of resistance to Bd of some amphibians in Panama (Varela et al. 2018). The *Atelopus varius* in comparison to the other species that have resisted the pathogen, may be the most vulnerable species (Woodhams, Voyles, Lips, Carey, & Rollins-Smith, 2006). Ecological changes in habitats may prevent immunity in amphibians (Rollins-Smith et al., 2002). This means that habitat may be affected by a series of bioclimatic factors that could change how amphibians respond to diseases.

There are limitations to these studies in the Costa Rican and Panamanian tropical rainforests. While research hopes to find a bacterium to fight Bd, scientists and officials around the world will need to implement a mitigation strategy to prevent infectious pathogens that can negatively affect wildlife and public health.

We understand that amphibians are being threatened by a chytrid fungus, *Batrachochytrium dendrobatidis* (Bd). We still don't understand if the cause for their extinction is related to the interaction of Bd with climate change. And what we still don't understand is my research question: If Bd is one major factor of amphibian population decline and extinction, what makes it so prevalent? How can Bd impact the aquatic habitat in the tropical rainforest? What is the relationship between the amphibian chytrid fungal disease and climate change? This is why I will attempt to first perform forensic ecology of each *Atelopus* species in Costa Rica and Panama, examine the phylogenetic relationship of *Atelopus* with species diversity loss due to Bd, and analyze climatic variables from the late 1980s to 2018 to determine if Bd prevalence in Costa Rica and Panama is connected to climate change.

CHAPTER 3 Methods

3.1 Forensic History of the *Atelopus* Species in Costa Rica and Panama

For my first method, I described my attempt to collect all available sources to create a detailed history of each *Atelopus* species in Costa Rica and Panama. My aim was to provide a detailed narrative of the timeline of appearance of Bd, timeline of decline and in some cases recovery, and important existing knowledge of location, life history, habitat, and climate variables in order to see whether there are discernable patterns across these species that might help us understand the cause or causes of extinction. I collected observations from La Marca et al. (2005), the IUCN Red List of Threatened Species (2019), data and many other sources with information on taxonomic identification, geographic distribution, elevation range, current and past estimates of abundance, current population status, last documented records, habitat and ecology, threats and conservation actions. Lips (2008) provided a 30,000-foot view of the epidemic wave. I wanted to provide a closer view (5,000 foot) for Panama and Costa Rica. Perhaps by looking at the extinction episode at a smaller geographical scale and over a longer time period, I could assess potential causes for decline and recovery. I determined the actual coordinates of each *Atelopus* species in Panama and Costa Rica and calculated the mean elevation of each *Atelopus* species occurrence.

3.2 *Atelopus* Species Phylogenetic Tree

For the second part of my research, I collected the 16S ribosomal RNA gene sequence data from all *Atelopus* species from Central and South America from the National Center for Biotechnology (NCBI) to determine the connection between genetics and Bd . There was no16S ribosomal RNA gene sequence data from *Atelopus senex*. I input each *Atelopus* species sequence data as text and uploaded it to the Seaview Sequence Alignment and Phylogenetic Tree Building software. The DNA sequences of the *Atelopus* species were aligned and a phylogenetic tree was constructed. The IUCN (2019) status of each *Atelopus* species was supplemented as a legend labeled in different colors to illustrate the *Atelopus* threatened status, which are extinct (red), extinct in the wild (orange), critically endangered (golden), endangered (yellow), vulnerable (light blue), near threatened (purple), least concerned (green), data deficient (dark blue), and not evaluated (gray).

3.3 Correlation Between Bd Prevalence in *Atelopus* Species and Climatic Variables in Panama and Costa Rica

For my third method, I used the distribution species collection data from the IUCN that included duplicates, which makes the data spatially biased. Here, I analyzed how the Worldclim 19 climatic historical bioclimatic variables from 1970 to 2000 in Costa Rica and Panama related to a species abundance, based on unbiased datasets for 8 *Atelopus* species using RStudio.

I identified the coordinates of the location data of each *Atelopus* species accurately to allow RStudio to recognize the *Atelopus* species data. To analyze unbiased data, I removed points with no location data, points that were not in the right geographical location, for example, I only needed coordinates for Panama and Costa Rica. I also removed duplicates (observations from the same location), and created data frames for points with different coordinate systems, and converted them to WGS 1984.

I focused on the 2 most common bioclimatic variables in Panama and Costa Rica. For BIO1 (Mean Annual Temperature) to produce the bioclimatic response model using dismo. I analyzed the probability of the *Atelopus* species occurrence in their home range based on the temperature per year. For BIO12 (Mean Annual Precipitation), I analyzed the probability of the *Atelopus* species occurrence in their home range based on precipitation per year. The bioclimatic temperature variables in BIO1 are shown in (°C x 10). The *Atelopus* species habitat suitability model included values from 0 for unsuitable habitats to 1 for suitable habitats.

CHAPTER 4 Results

4.1 Forensic History of the *Atelopus* species in Costa Rica and Panama

In this chapter, I am presenting the history of Chytridiomycosis, the skin infection caused by Bd that has affected the *Atelopus* species *(A. chiriquiensis, A. varius, A. limosus, A. zeteki, A. glyphus, A. certus, A. chirripoensis, and A. senex)* in the Panamanian and Costa Rican region. This narrative describes the assessment of the native Panamanian and Costa Rican *Atelopus* species distribution and population decline caused by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd) (La Marca et al., 2005; Lips et al. 2008). I present a summary of the individual *Atelopus* species and the Panama – Costa Rica region at the end of Section 1 in Tables 4 & 5, and Figure 11.

4.1.1 Chiriquí Harlequin Frog (*Atelopus chiriquiensis)*

The *Atelopus chiriquiensis* has been listed as critically endangered by the International Union for Conservation of Nature's Red List of Threatened Species (IUCN, 2019), because of a severe loss in its population, possibly to be more than 80% over 21 years. The probable cause of the loss of most of the population is due to Chytridiomycosis. This species was last seen in the 1990s (IUCN, 2004). The status of *A. chiriquiensis* is considered to be extinct in the wild with no captive population (Lewis et al., 2019).

The *A. chiriquiensis*, a diurnal forest frog, is closely related to *A. varius*, the harlequin frog from Monteverde (Lips, 1998), is found in streamside in the lowland forests between the Cordillera de Talamanca-Chiriquí of Costa Rica (1,800-2,500 m in elevation) and Western Panama (1,400-2,100 m in elevation) (Savage 2002). This species has not been seen in Costa Rica since 1996 (LaMarca et al., 2005), and it is believed to be extinct in Costa Rica and Panama (Lips et al., 2010).

This *A. chiriquiensis* population at one time was widespread in Costa Rica and Panama (Lips et al., 2010). Surveys were conducted in search of this species, but it has not been seen in their habitats since 1996 (LaMarca et al., 2005). For example, Karen Lips surveyed Las Tablas, Costa Rica in 1990, and 1991 when the species were most abundant (Lips, 1998). However, in 1992 and 1993, the species began to decline, and by 1994 and 1996, Lips did not find any *A. chiriquiensis* (1998). In their last assessment in August 2007, the IUCN described no new detection of the *A. chiriquiensis* (Lips et al., 2010), and according to Lewis et al. (2019), there is no *A. chiriquiensis* in captivity (2019).

The *Atelopus chiriquiensis* faced threats over the years and have been observed and documented (Lips et al., 2003). A possible cause of the extinction of this species in Costa Rica is due to *Batrachochytrium dendrobatidis* (Bd) (Lips, 1998), which was confirmed in this species in 1993 and 1994 (Lips et al., 2003). Introduction of predatory trout, and general habitat loss both outside, and within protected areas, are also threats to remaining populations. Climate change is considered to be a possible threat.

El Parque Nacional Chirripó (Chirripó National Park) and Parque Internacional La Amistad (La Amistad International Park) are protected areas that cover the *A. chiriquiensis* home range. If surviving species are found, there would be a conservation plan to protect this species against Bd (Lips at al., 2010).

4.1.2 Variable Harlequin Frog *(Atelopus varius)*

The IUCN, 2020 listed the *Atelopus varius* as Critically Endangered in the wild. More than 80% of its population over 21 years between 1987 and 2007 has suffered a severe decline (Lewis et al., 2019). The population decline of this species is due to Chytridiomycosis (Baillie et al., 2004). The population decline has been significant in their suitable habitats at above 1000 m of elevation (LaMarca et al. 2005). A small population of *A. varius* has been rediscovered in Costa Rica and Panama areas with Bd prevalence (Lewis et al., 2019). A large population of *A. varius* was found in the Bd prevalent lowland forests, in Donoso, in the Panamanian province of Chiriquí (Lewis et al., 2019). The status of this species in captivity is secure (Lewis et al., 2019). There are 160 *A. varius* in captivity in American zoos and 24 *A. varius* in Panama that comprises 8 highland frogs, and in 2016, 16 lowland frogs that were collected from Donoso (Lewis et al., 2019).

The *A. varius* are terrestrial, diurnal forest frogs. They are found in both Atlantic and Pacific highland areas of the cordilleras of Costa Rica throughout El Copé in Central Panama (Richards and Knowles 2007), up to 2,000 m in elevation (Savage, 1972). It is also found in remote lowland areas at 16m in elevation (Pounds et al., 2010). There is a probability that a population of *A. varius* occupies an unexplored montane forest in Costa Rica (Ryan, Berlin, Gagliardo, & Lacovelli, 2005). The Panamanian and Costa Rican *Atelopus various* have genetic differences. As a result, there are a wide variety of this species dispersed in small populations (Lewis et al., 2019).

The Harlequin frogs were widespread in Costa Rica (Pounds et al., 2010) and have decreased over the years. However, the severe loss was recorded in Monteverde in 1988, and the species was considered extinct in Costa Rica by 1996 (Lips et al., 1998). According to the IUCN 2012, 80% of the *Atelopus* species are critically endangered, resulting in a significant number that may be extinct (González-Maya et al., 2018). There are surviving populations of Harlequin frogs occupying the Central Panama region after the Chytrid fungus wave (Richards and Knowles, 2007). The Harlequin frogs might have developed a resistance to Chytridiomycosis. Their survival in the wild can help other amphibians resist the disease if they share similar phylogenetic lineage.

In 2003 a small population was rediscovered in Costa Rica. A group of scientists led by Ron Gagliano, from the Atlanta Botanical Garden found 3 Harlequin frogs on the Pacific coastal range near Quepos (Ryan et al., 2005). Gagliano conducted a second survey in 2005 but did not find additional populations (2005). However, another type of *A. varius* population was discovered in the Talamanca Mountains in 2008 and Quepos in 2015 (González-Maya et al., 2013). Although there is not enough literature with evidence to support claims about the *A. varius* population in the Provinces of Veraguas, Coclé in Central Panama and Colon in the Central side of Panama near the Caribbean Sea (González-Maya et al., 2013), some populations including the *A. varius*, have declined (Lips, 1999). In Chiriquí Province, Western Panama, a significant number of *A. varius* died between 1996 and 1997 (Lips, 1999). The site was surveyed again in 1998, but the species did not prevail (Ibañez, 1999). However, in Coclé, Central Panama, another *A. varius* population has persisted Bd at the same location where the massive decline occurred (Voyles et al., 2018). In Costa Rica, a population of A. varius has been rediscovered and perhaps resisting Bd (Ryan et al., 2005).

The *A. varius* is a terrestrial species of humid lowland and montane forest; specimens recorded at lowland rainforest localities were all found along high-gradient, rocky streams, in hilly areas (Savage 2002). It is associated with small fast-flowing streams and is often found along the banks and sitting out on rocks in streams; at night they sleep in crevices or low vegetation. They were previously present and densely populated during the dry season, from December to May in humid habitats (Savage 2002). Eggs are laid in water and are probably attached to rocks, and its larva disperse through streams (Richards-Zawacki, 2009).

In the late 1980s, pet trade, habitat loss, invasive species such as trout (*Onchorhynchus* and *Salmo*) and American bullfrog (*Rana catesbeiana*) (LaMarca et al., 2005), and climate change caused the *A. varius* population decline (González-Maya et al., 2013). However, chytridiomycosis is the major cause of amphibian loss which has led to catastrophic population declines in many other montane species of *Atelopus* (Lips, Reeve, & Witters, 2003). Museum specimens of this species have been found to have chytrid fungi. The *Atelopus varius* began to vanish in Monteverde then its extinction in the Tilarán Mountains in Costa Rica in 1992 (González-Maya et al., 2013). One specimen collected in 2003 from the only known site at which the species survives in Costa Rica tested positive for chytrid infection, and the disease was also confirmed in individuals in 1986, 1990, 1992 and 1997 (LaMarca et al., 2005). Other threats include the parasitic fly larvae that pray on this *Atelopus* species (González-Maya et al., 2013), habitat loss due to the destruction of natural forests, and predation by introduced rainbow trout. Besides Bd, another major threat is the unlawful collection for the pet trade if the location of this species population is exposed (González-Maya et al., 2013). The only known site in Costa Rica is under serious threat of a landslide that could potentially destroy the entire stream section where they are presently found. It was collected by the thousands in the 1970s and shipped to Germany as part of the international pet trade. In Panama, anthropogenic activity such as the creation of dams, invasive predatory fish, water pollution by agricultural runoff are potential cause of the *Atelopus varius* population decline (Richards-Zawacki, 2009).

The *A. varius* is present in three undisclosed protected areas in Panama. This species was previously found in a number of Costa Rican protected areas in Northeast of San Vito, within the Las Tablas Protected Zone, La Amistad Biosphere Reserve (González-Maya et al., 2013). This location is a conservation area that covers restored forests with steep slope streams (2013). *Ex-situ* conservation actions are now needed to ensure the future survival of this species, and a captive-breeding program has been started. In Panama, the species survival program imported the A. varius to zoos in the United States in 2001 to ensure the population of this species (Lewis et al., 2019). Then in 2009, Zoo New England, Cheyenne Mountain Zoo, Houston Zoo, Smithsonian National Zoo, The Smithsonian Tropical Research Institute (STRI), and Defenders of Wildlife collaborated to create the Panama Amphibian Rescue and Conservation (PARC) Project, located in Gamboa, Panama, to ensure the population of 12 amphibian species, including the *A. varius* (2019).

4.1.3 Limosa Harlequin Frog *(Atelopus limosus)*

The IUCN, 2019 listed the *Atelopus limosus* as Critically Endangered and its population is decreasing (IUCN, 2019). Its population may have started to decline in 2009, as this species was tested positive for Bd (Lewis et al., 2019). There is continuing decline in the extent and quality of its forest habitat in Panama. A. limosus population will probably have approximately 80% loss for 21 years (2019).

The *A. limosus* is endemic to Colón, in the eastern Atlantic side of central Panama, but this species has a very extensive distributional range. The *A. limosus* is present in low-altitude at a 10-730m in elevation (Lewis et al., 2019), in the Atlantic slope of several areas throughout the Panama Canal (Ibañez et al., 1995), which are Coco Solo, Brazo del Medic, the Chagres River, Madden Lake, and Gatún Lake in the province of Colón and near Boquerón in the province of Panama (1995), and in higher elevation sites including Cerro Bruja, Cerro Brewster, Valle de Mamoní, Cocobolo Nature Reserve and Nusagandi within the Comarca Kuna Yala, which is an indigenous territory in the Atlantic side of Panama (IUCN, 2019).

The *A. limosus* population was abundant in Santa Rita, in Colón Province in 2000 only one individual was seen in December 2002 until 2009 when Bd was discovered in Chagres National Park (Lewis et al., 2019). Since 2009, this species has suffered a rapid population loss in higher elevation areas including Cerro Bruja, Cerro Brewster, Sierra Llorona, and the population in the lower elevation suffered a slight decline (2019). Since 2018, the *Atelopus limosus* is still present in small populations in the Cocobolo Nature Reserve located within the Mamoní Valley Preserve in the East side of the Panama Canal

(Lewis et al., 2019), although some Limosa harlequin frogs have been found positive with Bd (IUCN, 2019). The status of the *A. limosus* in captivity is secure with 26 frogs that have been bred in captivity, but tadpoles from only 20 adult pairs have succeeded to adulthood and created the current captive *A. limosus* community (Lewis et al., 2019).

The *A. limosus* is a terrestrial species of tropical lowland forest (Ibañez et al., 1995) This species is active during the day, and is present in rocky river streams in cloud forest (1995). The A. limosus Breeding and larval development takes place in forest streams (Ibañez et al., 1995). The skin color of this species resembles the surface of rocks and the streams, which serves as a disguise to protect themselves from predation (1995). The A. limosus in the lowland differ from the A. limosus in the highland (Wilson, 2014). The lowland type is brown with yellow nostrils and fingertips, while the highland type has green and yellow with a black wide V-shaped pattern on the back of its body (2014). The average generation interval for this species is 7 years (IUCN, 2019).

Although deforestation of habitat for agricultural use and general infrastructure development, water pollution, stream sedimentation (IUCN, 2019), and gold mining (Ibañes et al., 1995), are considered major threats to this species a significant quantity of *A. limosus* population have declined from their home range due to the chytrid fungus, Bd, which appears to be more prevalent at high elevations sites (Lewis et al., 2019). However, in a 2018 survey, the *A. limosus* in the wild have been persisting Bd at several sites within the Mamoní Valley (2019). The population of *A. limosus* in the lowlands have not been significantly affected by Bd. This population can prevent Bd infections due the warmer temperature at lower elevations sites (Flechas et al., 2012). The skin microbiome on the *A. limosus* may be a significant influence in preventing chytridiomycosis (2012).

A significant population of *A. limosus* has been documented from Parque Chagres National Park and Comarca Kuna Yala, but habitats within the Mamoní Valley Preserve are high priority for the Panama's National Amphibian Conservation Action Plan (ANAM) and PARC established to support habitat protection and research (Gratwicke et al. 2016).

A reintroduction of *A. limosus* has been initiated. In 2017, PARC started a release trial of 90 *A. limosus* at Mamoní Valley Preserve (Lewis et al., 2019). Scientists used a direct release to the wild and a soft release approach with a small frog group, which means that captive frogs would remain ex-situ for 30 days before their release to the wild (2019). Tracking radio transmitters were attached to some frogs, however most of them scattered out of the monitoring range after their release and contributed to a small recapture percentage (2019). Efforts to reintroduce captive frogs in Mamoní Valley Preserve continues to understand the outcome of this species in Bd prevalent sites as a tool to mitigate chytridiomycosis.

4.1.4 Panamanian Golden Frog *(Atelopus zeteki)*

A. zeteki. Source: E. Monk, STRI

The *Atelopus zeteki* is culturally significant to the people of Panama (Markle, 2012), with a history dating back to the Mayan civilization (2012). The IUCN listed the *Atelopus zeteki* as Critically Endangered, Possibly Extinct in the Wild (IUCN, 2019). Karen Lips reported a significant population of Panamanian golden frog in 1992

(Markle, 2012). In 1996, Lips reported that this species has suffered a severe decline (2012). The *Atelopus zeteki* have been rescued and relocated to captive breeding centers (Zippel, 2002), to protect them from Bd (Lips, 2006). The last *Atelopus zeteki* in the wild was seen in 2009 (Lewis et al., 2019). It is estimated that more than 80% of the *A. zeteki* population has vanished over the last 10 years, probably due to chytridiomycosis (IUCN, 2019).

The *Atelopus zeteki*, an endemic species to Panama, was present in the rainforests and cloud forests east of the main Tabasará ridge in Coclé in low elevations at 335 m, in middle elevation around El Valle de Antón in Coclé at 760 m (Savage, 1972), to high elevation at 1,315 m (Stuart et al. 2008), in Cerro Campana (Richards and Knowles 2007). The A. zeteki is limited to these areas and has not distributed from its home range.

The *Atelopus zeteki* was abundant at a number of sites in north of El Copé within the Omar Torrijos National Park in the Coclé Province (Lips, 2006), but this species is now extinct in the wild (IUCN, 2019). Lips surveyed these areas for 4 years from 2000 and July 2004 (2006). By September and October 2004, Populations have been declining due to chytridiomycosis, and the well-known El Copé population collapsed, and vanished (2006). Consequently, A fraction of the last *A. zeteki* population on Cerro Campana and in the El Valle de Antón have disappeared due to Bd (McCaffery et al. 2015). The last wild *Atelopus zeteki* was seen in 2009 (Lewis et al., 2019). The status of the *Atelopus zeteki* in captivity is secure (2019), PARC has collected 4 wild adults highland *Atelopus zeteki* in Panama. The Golden Frog Species Survival Program in the United States helped breed 32 captive *Atelopus zeteki*, which include 12 lowland frogs and 20 highland frogs *(Lewis et al., 2019).* The 2 groups of captive frogs reproduced more than 1300 adult frogs in captivity (2019).

The *A. zeteki* is a terrestrial species of tropical montane wet forest, and montane dry forest (Poole, 2006), with breeding and larval development taking place in forest streams (Savage 1972). The tropical montane wet forest is larger and more dispersed in and along the streams (up to 3m above the ground). The habitat typically includes waterfalls and large boulders covered with moss that they utilize as visible territories. These frogs sleep on big leaves at night (Poole, 2006). The *Atelopus zeteki* from the tropical dry forest are smaller than the highland golden frogs and more visible on the forest floor (2006). The *Atelopus zeteki* uses its skin secretions to protect themselves from predators (Savage 2002).

The major threat is chytridiomycosis, which has led to catastrophic population declines in many other species of the *Atelopus* species (Pounds et al., 2006; McCaffery et al., 2015, Becker et al., 2015). In the 1960's the *Atelopus zeteki* was collected severely for pharmaceutical purposes in Europe and the United States (La Marca et al., 2005), and pet trade (Lewis et al., 2019). The first declines caused by Bd were documented in 2004 in el Cope, near El Valle de Anton, Central Panama (Poole, 2006). Another threat to the *Atelopus zeteki* is habitat loss due to deforestation, as well as water pollution (La Marca et al., 2005). Sedimentation significantly affected river streams near El Valle de Antón, due to road constructions (Lewis et al., 2019).

Although the *Atelopus zeteki* is protected in areas of Altos de Campana National Park in and Omar Torrijos Herrera National Park by national legislation decree No. 23 of January 30, 1967 (Zippel et al., 2006), it is possibly extinct in the wild (IUCN, 2019).

PARC has started a captive-breeding program with zoos in the United States, to create stable populations in captivity (Poole, 2006). However, the reintroduction of this species in the wild is not possible until existing threats can be addressed. Another program from PARC is El Valle Amphibian Conservation Center, (EVACC), which is an *in-situ* program with 2 locations at El Valle de Antón in the Western Panama and at Gamboa (ARCC), near the Panama Canal, which has an amphibian exhibition and a research center dedicated to the efforts to mitigate Bd (Lewis et al., 2019).

4.1.5 Pirre Harlequin Frog *(Atelopus glyphus)*


A. Glyphus. Source: M. Guerra, STRI

The IUCN, 2019 listed the *Atelopus limosus* as Critically Endangered and its population is decreasing (IUCN, 2019). The population of this species was considered stable in 2002 (La Marca et al., 2005). In 2015, a single *Atelopus glyphus* frog was first found positive for Bd (Lewis et al., 2019). Studies show that after Bd is detected in a species, the entire population experience a severe decline (Lips et al., 2008). In 2018, another *Atelopus glyphus* individual was detected positive for Bd (Lewis et al., 2019) The population of the *Atelopus glyphus* will probably to decline 87% over the next 21 years, suggested from declines in other *Atelopus* species in the same region, due to Bd (Gratwicke et al., 2016).

The *Atelopus glyphus* species is present in Darién, eastern Panama, in the mountainous site of Serranía de Pirre (Savage, 1972) at a mean elevation of 1192 m. Previous assessment by the IUCN stated that the *Atelopus glyphus* was also found in the Chocó of Colombia, but these records have not been confirmed (IUCN, 2019).

Consequently, the home range of this species has been restricted to the Panamanian site within the Darien National Park and the updated extent of occurrence is 381 km^2, (2019)

The *A. glyphus* population is decreasing. This species was considered to be common within its home range (Savage, 1972). It was still abundant in September 2002 in the Serranía de Pirre, above Cana, in Darién, eastern Panama (Ibañez, 2015). The status of the *Atelopus glyphus* in captivity is almost secure (Lewis et al., 2019). 18 of the 20 founders *Atelopus glyphus* produced surviving captive descendants, resulting in being below population goals required by the Amphibian Ark, which has 350 adult amphibians in captivity (2019). The goal of the Amphibian Ark was to reach up to 500 adult frogs including captive-bred offspring (2019). In 2015, scientists reported dead *Atelopus glyphus* frogs were positive for Bd, in the first field observations (Lewis et al., 2019). The last surveys to Cana in January 2018 recorded only a single individual with three days of searching (2019). There is no recent information about the *Atelopus glyphus* population in the Darien area as the roads are inaccessible due to a political situation by the guerrilla movement the Revolutionary Armed Forces of Colombia (FARC) in the eastern Panamanian border (Ibañez, 2018).

The *Atelopus glyphus* is a terrestrial species of tropical mountainous rainforest at 884 -1,500 m in elevation (Savage, 1972). Amphibians living in riparian, montane habitats stay within their own home range (Stuart et al., 2008). The *Atelopus glyphus* species breed in forest streams (Ibañez, 2004) There is no information on whether or not this species can survive in degraded habitats (2004). The *Atelopus glyphus* can live up to 7 years in captivity (Gratwicke et al., 2016)

The most significant threat to the *Atelopus glyphus* is chytridiomycosis, due to the chytrid fungus Bd that caused a devastating population declines in *Atelopus* species (Pounds et al., 2006; McCaffery et al., 2015, Becker et al., 2015). About 80% of *Atelopus* species have suffered a decline in their population (La Marca et al., 2005). Other threat to this species is habitat loss due to agricultural development (including the planting of illegal crops by the FARC guerilla), logging, and human settlement, and pollution resulting from the spraying of illegal crops (Ibañez, 2018).

The *Atelopus glyphus* has been recorded from two protected areas: Darién National Park (a World Heritage Site) in Eastern Panama and Parque Nacional Natural los Katíos in Colombia. Considering the severe risk of Bd infection on *Atelopus* species, an ex-situ population has been established by PARC (Gratwicke et al., 2016), however the conservation site in Cana has been difficult to access due to the FARC guerrilla (Ibañez, 2018).

4.1.6 The Darien Stubfoot Toad *(Atelopus certus)*


A. certus. Source: R. Ibañez, STRI

The *Atelopus certus* is listed by the IUCN as Critically Endangered (IUCN, 2019). It is inferred that this species will probably undergo a population decline due to Bd, estimated by La Marca et al. (2005), to be more than 80% over the next 21 years, recognized from declines in other high altitude *Atelopus* species in the same region (IUCN, 2019).

The *Atelopus certus* is endemic to the eastern slope of Cerro Sapo in Darién Province of Panama at 50 -1,150 m of elevation (Savage, 1972), located south west outside of Darien National Park (Lewis et al., 2019).

The *Atelopus certus* species is present within its small range. There were less frogs documented than anticipated in the last survey in 2016, in Darién (Lewis et al, 2019). No single *Atelopus certus* was found positive for Bd during the survey (2019). It is not clear if the decline of this species is related to Bd, or the source was the drought caused by El Niño in 2016 (2019). The status of the *Atelopus certus* in captivity is secure. From the 28 captive *Atelopus certus*, 22 produced adult descendants and the captive population is about 350 adult frogs (2019).

The *Atelopus certus* is a terrestrial species of tropical montane and submontane forest (Savage, 1972). Breeding and larval development takes place in forest streams. The *Atelopus certus* species breed in forest streams (Ibañez, 2004) The eggs are large and unpigmented (Duellman & Lynch, 1969). Tadpoles have a large suction disc on the belly, used to cling to rocks and pebbles in streams (1969). This and can live up to 7 years in captivity (Gratwicke et al., 2016).

There are no records of population decline for the *Atelopus certus* species due to Bd. However, the *Atelopus* species is significantly vulnerable to Bd, with the highest mortality rates (Stuart et al., 2008; Gratwicke et al. 2016). The *Atelopus certus* is expected to decline about 80% of its population in the next 3 generations (La Marca et al., 2005). Although this species is present in lower elevation habitats, population loss has been recorded in low elevations in the *Atelopus limosus* species (Lewis et al., 2019).

Other threats to the *Atelopus certus* outside of its protected range, Darien National Park, may be deforestation of habitat for agricultural use water pollution logging, and human settlement. Due to the political situation by the FARC guerrilla in the eastern Panamanian border there is no accessibility outside Darien National Park (Ibañez, 2018).

There is a conservation action in place for the *Atelopus certus*. The majority of its range is within the Parque Nacional Darién, with 91-100% of the population believed to be protected. In 2010, PARC set an expedition to the Darien area and brought back a founding population of *Atelopus certus* to begin an *ex-situ* conservation program at the Gamboa Amphibian Research and Conservation Center where the breeding process was successful (Gratwicke et al. 2016). The *Atelopus certus* is considered a priority species by the Panama's National Amphibian Conservation Action Plan (ANAM 2011).

4.1.7 The Chirripó Stubfoot Toad *(Atelopus chirripoensis)*

The *Atelopus chirripoensis* is listed by the IUCN as Critically Endangered, Possibly Extinct (IUCN, 2013). In 1980 one single individual was discovered (Savage & Bolaños, 2008). No single *Atelopus chirripoensis* has been found after many surveys following the discovery of this species, and it is believed that this species is extinct (2008). However, the IUCN indicates that if the species is still existing, there would be less than 50 and 160 adult frogs (IUCN, 2013).

Only one individual of *Atelopus chirripoensis* species has been seen 4 km north of the summit of Cerro Chirripó, Chirripó National Park in Costa Rica (Savage & Bolaños 2008). Cerro Chirripó elevational range is 3,400-3,500 m. Surveys in this area suggest that this species would be endemic to Cerro Chirripó (IUCN 2013).

In March of 1980, a Costa Rican biologist, Luis Gómez, collected a single frog was from a breeding accumulation of *Atelopus chirripoensis* north of Cerro Chirripó Grande in Costa Rica. Although many surveys have been conducted at the original site between 1980 and 1985, the *Atelopus chirripoensis* species has not been seen since (Savage and Bolaños 2008).

The *Atelopus chirripoensis* was present in high altitude grassland and shrubland qualified as Tropical Subalpine Pluvial Paramo region (Savage & Bolaños 2008). Dr. Gómez stated that there were many frogs of *Atelopus chirripoensis* species when he collected a single individual in 1980 (2008). The frog was found among a number of small shallow ponds that evaporate each season (2008). Although there is no information on its reproductive biology, it is presumed to breed by larval development in temporary ponds as observed by Dr Gómez (2008).

The *Atelopus chirripoensis* species has no records of population decline due to Bd (IUCN, 2019). The habitat of the species is not threatened because it is located in a remote, well-protected Chirripó National Park (Savage & Bolaños 2008). Cerro Chirripó is within the Talamanca mountain range, where Bd has been recorded (González-Maya, et al., 2013). Therefore, it is possible that the *Atelopus chirripoensis* species has disappeared seen since its discovery in 1980 due to Bd (IUCN, 2013).

The *Atelopus chirripoensis* was known to inhabit a protected within Chirripó National Park, which is a well-protected area, and adjacent highland areas are Las Tablas Protected Zone, La Amistad Biosphere Reserve (González-Maya et al., 2013). Surveys are needed to detect the existence of and threats to this *Atelopus* species (IUCN, 2013).

4.1.8 Pass Stubfoot Toad *(Atelopus senex)*

A. senex. Source: University of Kansas

The *Atelopus senex* is listed by the IUCN as Critically Endangered (Possibly extinct) because of a severe decrease in its population, estimated to be more than 80% over the last 21 years (La Marca et al., 2005), It is presumed from the evident loss of most of the *Atelopus senex* population, probably due to Bd (Bolaños, 2008).

The *Atelopus senex* species was present in high altitude regions of the rainforest in the Central Valley and Talamanca Mountain Range in Costa Rica from 1,100-2,200 m in elevation, in the headwater basin of Rio Grande de Orosi river, in only 3 isolated sites, 1 in the Barva volcano region with a lower elevation rainforest, and 2 on the extreme northern slopes of the Cordillera de Talamanca in Cedral Mountain and Reventazon basin which are premontane rainforests (Savage, 1972).

The *Atelopus senex* population was abundant on the slopes of Barba volcano but is now believed extinct there (Savage 2002). Although there were many surveys conducted, the last time the *Atelopus senex* species was seen was in 1986. This species experienced a population loss in 1987-1988, and did not recovered (Savage, 2002). Future surveys are needed to confirm the extinction of this species although further searches are needed to finally confirm the extinction of this species (Bolaños, 2008).

The *Atelopus senex* inhabits and reproduces in stream margins in premontane rainforest and lower montane rainforest. It is a diurnal, stream-breeding species, and used to be found in great concentrations during the reproductive period from July to August (Savage 2002).

The *Atelopus senex* experienced a severe population decline between 1987 and 1988, from which it has not recovered (Savage, 2002). The major threat to the *Atelopus senex* may be chytridiomycosis, leading to a catastrophic population decline, as has affected many other high-altitude species of *Atelopus* (La Marca et al., 2005). The *Atelopus senex* may be extinct in the Volcán Barva region (Savage, 2002). Other threats to this species may involve climate change, collecting for the pet trade, and pollution (Bolaños et al., 2008).

The range of this species is protected by both Tapantí National Park and Braulio Carrillo National Park (Savage, 2002). However, this species is now believed extinct in the Braulio Carrillo National Park (Bolaños et al., 2008). There is no *Atelopus senex* in captivity, therefore further survey work is required to determine whether or not this species still persists in the wild (2008). Considering that Bd may have devastated the *Atelopus senex* species surviving individuals might need to be established as a captive population.

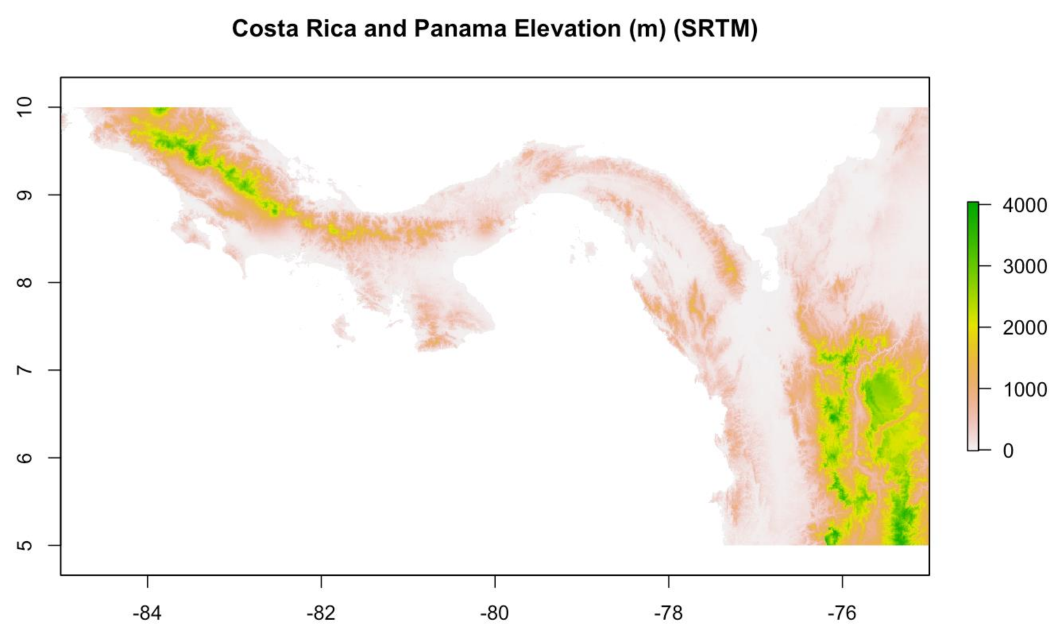
Table 4. Summary data on Atelopus *species. Prot. areas: Bd presence in protected areas. Yr. of last record: year of most recent record; Yr. Bd presence of Bd: year(s) documented; Hab. destr: occurrence of significant habitat destruction/ Status: Stable, Decline**

***Atelopus* species**	**Country**	**Elevational range (m)**	**Prot. areas**	**Yr. of last record**	**Yr. of Bd Presence**	**Hab. destr.**	**IUCN Status**
A. chirripoensis	Costa Rica	3400-3500	Y	1980	-	N	Unknown
A. senex	Costa Rica	1100-2200	Y	1986	-	N	Decline*
A. chiriquiensis	Costa Rica Panama	1400-2100	Y	1996	1993 1994	Y	Decline*
A. varius	Costa Rica Panama	16-2000	Y	2003	1986 1990 1992 1997	Y	Decline
A. zeteki	Panama	335-1315	Y	2004	2004	Y	Decline
A. limosus	Panama	10-730	Y	**2009**	**2009**	Y	Decline
A. certus	Panama	50-1150	Y	**2016**	-	N	Decline
A. glyphus	Panama	884-1500	Y	**2018**	**2015**	N	**Decline**

Table 5. Summary of Location, Coordinates Minimum, Mean, and Maximum Elevation of Atelopus *Species Occurrences.*

Species	Location	Longitude	Latitude	Min. Elev. (m)	Mean Elev. (m)	Max. Elev. (m)
A. chirripoensis	Chirripó NP, CR	-83.48	9.53	3400	3450	3500
A. senex	Volcán Barva, CR	-84	10	1960	2000	2040
	Reventazón Basin, CR	-83.46	10.23	1280	1300	1320
	Cedral Mt, CR	-84.14	9.84	2150	2285	2420
A. chiriquiensis	La Amistad, Intl. Park CR	-83.04	9.13	1400	1750	2100
A. varius	El Copé, El Valle, PAN	-80.62	8.67	758	1,036	1314
	Fortuna Forest, PAN	-82.16	8.71	700	1457	2213
	Monteverde, CR	-84.8	10.3	600	1200	1800
	Santa Fe, NP, PAN	-81.13	8.52	430	1197	1964
A. zeteki	El Copé, El Valle, PAN	-80.13	8.62	335	825	1315
A. limosus	Chagres NP, PAN	-79.47	9.45	10	370	730
A. certus	Darién NP, PAN	-78.35	7.94	50	600	1150
A. glyphus	Darién NP, PAN	-77.72	7.8	884	1192	1500

Figure 11. The mean elevation of Costa Rica and Panama ranges from 600 m to 3450 m.



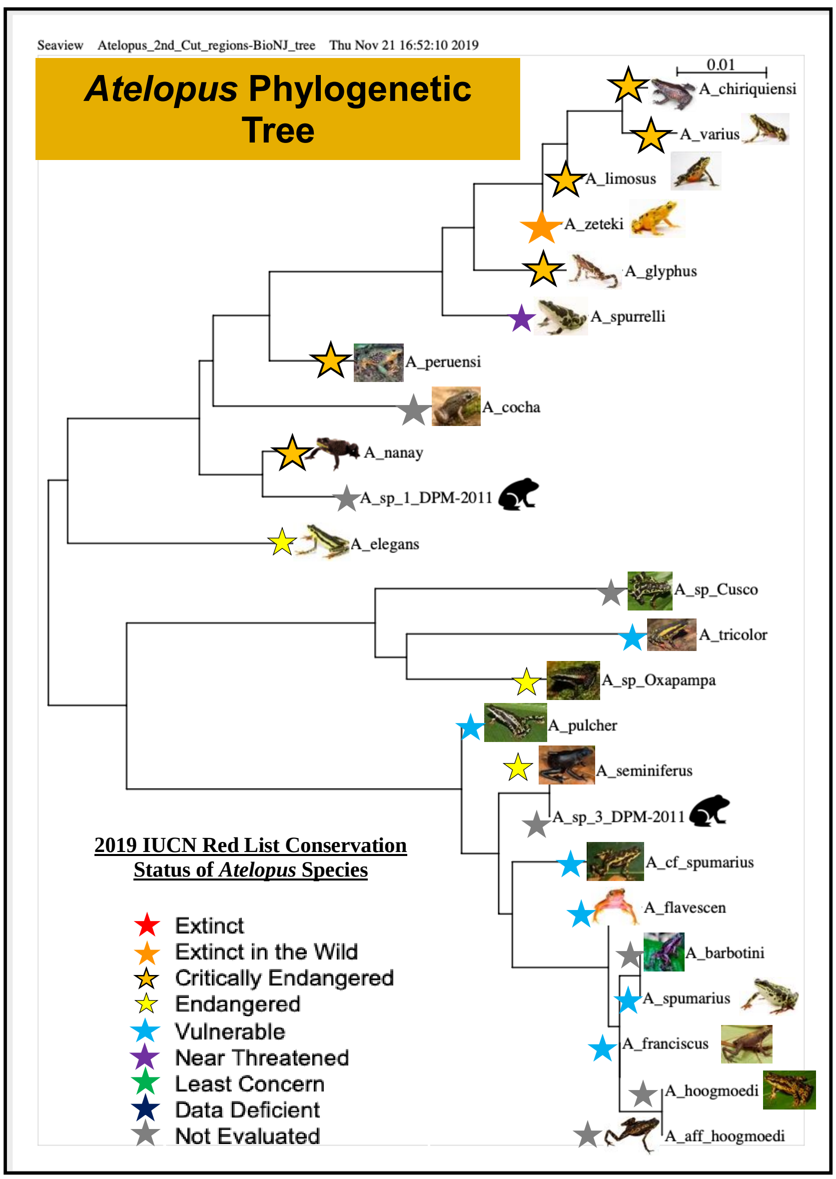
4.2 *Atelopus* Species Phylogenetic Tree

The *Atelopus* species phylogenetic tree shows the genetic variation of *Atelopus* species (for which genetic data were available, see *Methods*) from Central and South America (Fig. 12). The phylogenetic tree showed the top 5 *Atelopus* species indicates that 5 Costa

Rican and Panamanian. The *Atelopus chiriquiensis* and *Atelopus varius* occurred in Costa Rica and Panama. The *Atelopus limosus* is endemic to Colón on eastern Atlantic side of Central Panama, and the *Atelopus zeteki* and *A. glyphus* are also both endemic to Panama. I hypothesized that these *Atelopus* species geographic isolation may have caused the loss of their genetic variation and has endangered their immune responses to diseases.

The phylogenetic tree (Fig. 12) shows the IUCN red list assessment with the conservation status of each the *Atelopus* species of Central and South America.

Figure 12. Atelopus Species Phylogenetic Tree and IUCN Red List Assessment



4.3 Connection Between Bd Prevalence and Bioclimatic Variables in *Atelopus* Species of Panama and Costa Rica

In this result, I assessed the Bd connection with the loss of *Atelopus* species in Panama and Costa Rica based on bioclimatic variables (Table 6). Here, I presented the species distribution map, bioclimatic response plots, and the species distribution model using unbiased occurrence data of Chiriquí Harlequin Frog *(Atelopus chiriquiensis),* Variable Harlequin Frog *(Atelopus varius),* Limosa Harlequin Frog *(Atelopus limosus),* Panamanian Golden Frog *(Atelopus zeteki),* Pirre Harlequin Frog *(Atelopus glyphus),* Darien Stubfoot Toad *(Atelopus certus),* Chirripó Stubfoot Toad *(Atelopus chirripoensis),* and Pass Stubfoot Toad *(Atelopus senex).* These species were analyzed for climatic suitability based on 19 historical bioclimatic variables from 1970 to 2000, to visualize what the climate was like where the *Atelopus* species were known to be present during the Bd wave from 1980 to 2000. How did Bd affect the *Atelopus* species in connection to temperature, precipitation and elevation? The bioclimatic variables that most applied in Panama and Costa Rica are Bio1, temperature, and Bio12, precipitation (Fig.13).

*Figure 13. BIO1 (left) with temp. range from 7℃ to 20℃ per year (note, values in legend are ℃*10) and BIO12 (right) with precipitation. range from 2000 mm to 5000mm per year in Costa Rica and Panama.*

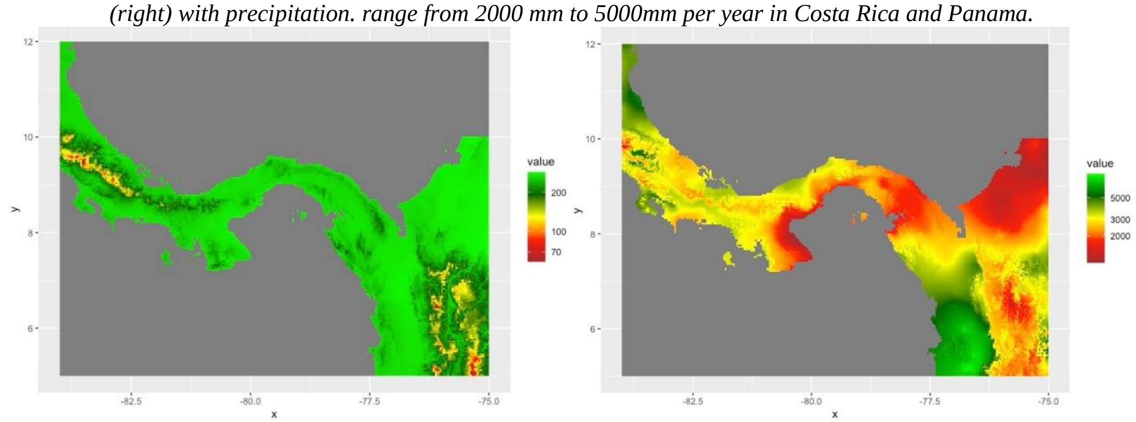


Table 6. List of 19 bioclimatic variables used in bioclimatic model development. Names and descriptions are in reference to the WorldClim, Hijmans, 2017.

Variable	Description	Temporal Scale
Bio1	Annual Mean Temperature	Annual
Bio2	Mean Diurnal Range (Mean of monthly (max temp - min temp))	Variation
Bio3	Isothermality (BIO2/BIO7) (×100)	Variation
Bio4	Temperature Seasonality (standard deviation ×100)	Variation
Bio5	Max Temperature of Warmest Month	Month
Bio6	Min Temperature of Coldest Month	Month
Bio7	Temperature Annual Range (BIO5-BIO6)	Annual
Bio8	Mean Temperature of Wettest Quarter	Quarter
Bio9	Mean Temperature of Driest Quarter	Quarter
Bio10	Mean Temperature of Warmest Quarter	Quarter
Bio11	Mean Temperature of Coldest Quarter	Quarter
Bio12	Annual Precipitation	Annual
Bio13	Precipitation of Wettest Month	Month
Bio14	Precipitation of Driest Month	Month
Bio15	Precipitation Seasonality (Coefficient of Variation)	Variation
Bio16	Precipitation of Wettest Quarter	Quarter
Bio17	Precipitation of Driest Quarter	Quarter
Bio18	Precipitation of Warmest Quarter	Quarter
Bio19	Precipitation of Coldest Quarter	Quarter

4.3.1 Chiriquí Harlequin Frog *(Atelopus chiriquiensis)*

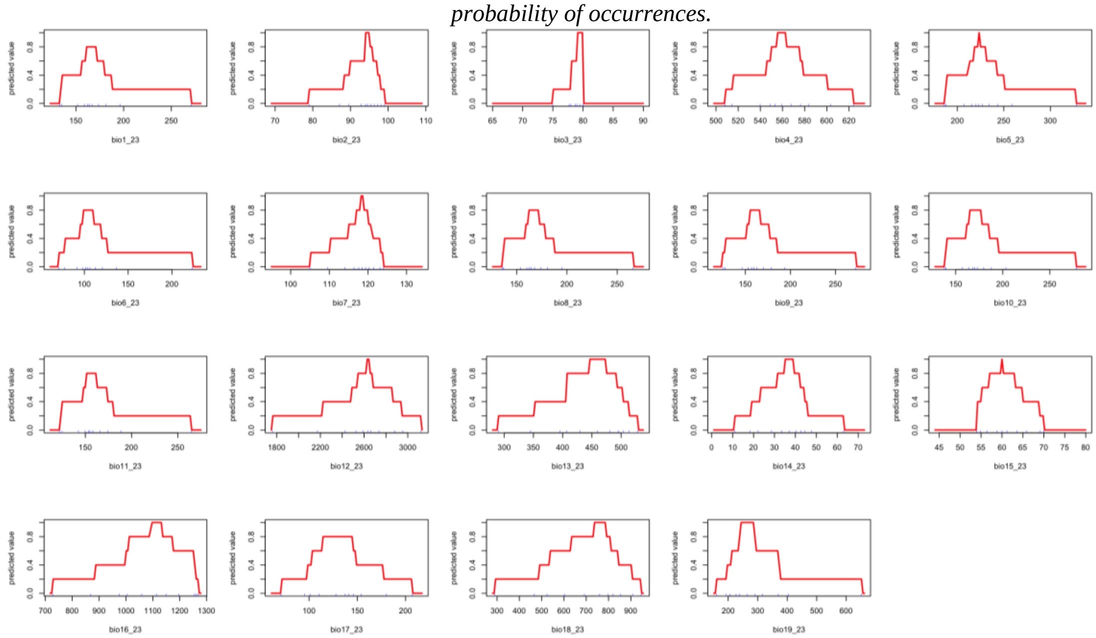
This is the preliminary map (Fig. 14) of the Chiriquí Harlequin Frog *(Atelopus chiriquiensis)* distribution with biased observation points to see how this species is distributed in the lowland forests between Eastern Costa Rica (mean elevation 2,150 m) and Western Panama (mean elevation 1,750 m).

Figure 14. Map of the Atelopus chiriquiensis Distribution in Costa Rica and Panama

The response plot (Fig. 15) showed how the probability of the *Atelopus chiriquiensis* occurrences (y-axes), from 0 to 1, differ with each bioclimatic predictors (x-axes). I focused on the 2 most common bioclimatic variables for Panama and Costa Rica, which are BIO1 (Mean Annual Temperature) and BIO12 (Mean Annual Precipitation). The response plot shows thresholds, and linear and nonlinear shapes.

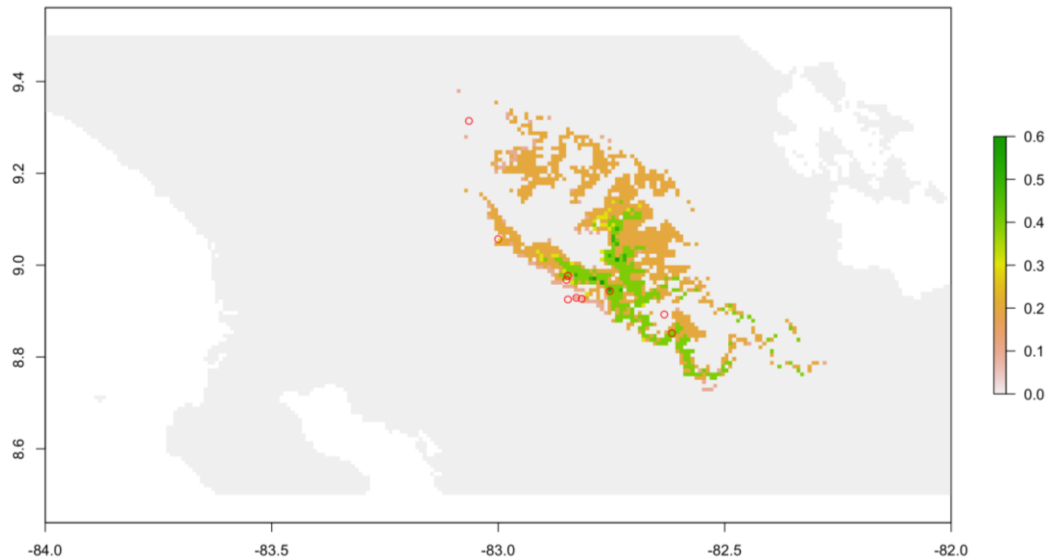
BIO1 showed that below the threshold of about 13°C (55 °F) of temperature per year, the probability of the *Atelopus chiriquiensis* occurrence was 0. The probability increases nonlinearly at about 14 °C (57 °F) per year, and the probability of this species occurrence at its peak at about 16 °C (61 °F) per year, was 0.8, then the probability of the *Atelopus chiriquiensis* occurrence plateaued at 18 °C (64 °F). Therefore, the probability of this species to occur in this range was between 14 °C (57 °F) and 18 °C (64 °F) per year. In BIO12, the probability of the *Atelopus chiriquiensis* occurrence below the threshold of about 1760 mm of precipitation per year was 0. The probability of this species occurrence increased nonlinearly at about 2500 mm of precipitation per year with a predicted value of 0.4 and reached its peak at 2680 mm of precipitation per year with a probability of occurrence of 1, then the probability of occurrence decreased nonlinearly at about 2900 mm per year with a probability of occurrence of 0.4. Therefore, the probability of the *Atelopus chiriquiensis* to occur in its range was between 1760 mm and 3120 mm of precipitation per year.

Figure 15. Atelopus chiriquiensis Response to Bioclimatic variables range of values based on the estimates of the probability of occurrences.



What the habitat suitability plot (Fig. 16) predicted about the *Atelopus chiriquiensis* distribution of suitable habitats at the border of Costa Rica and Panama was that 30% of this species observed had suitable habitats, but 70% of *Atelopus chiriquiensis* observed in places where the probability of occurrence was predicted to be low, below the actual observations in 3 areas with suitable habitats where the probability of occurrence was 0.4. However, there were also 7 areas with habitats where the probability of occurrence predicted to be below 0.2, which were not suitable for the *Atelopus chiriquiensis* to survive.

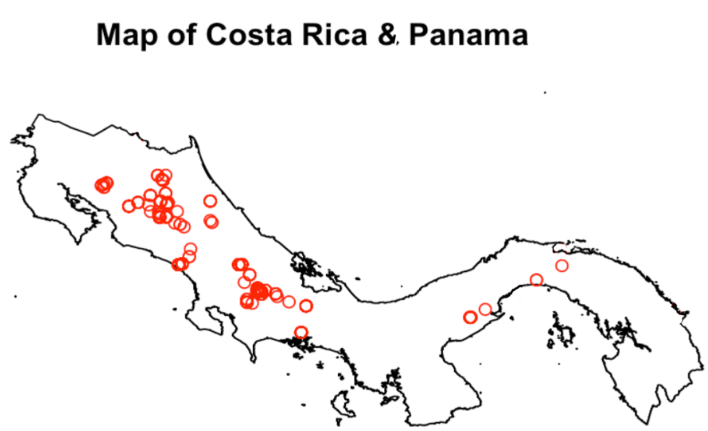
Figure 16. Predicting Recent Climatic Habitat Suitability on the Atelopus chiriquiensis range



4.3.2 Variable Harlequin Frog *(Atelopus varius)*

This is the preliminary map of the Variable Harlequin Frog *(Atelopus chiriquiensis)* (Fig. 17), distribution with observation points to see how this species is distributed in high montane areas of Costa Rica throughout El Copé in Central Panama with a mean elevation of 1223 m.

Figure 17. Map of the Atelopus varius Distribution in Costa Rica throughout Panama

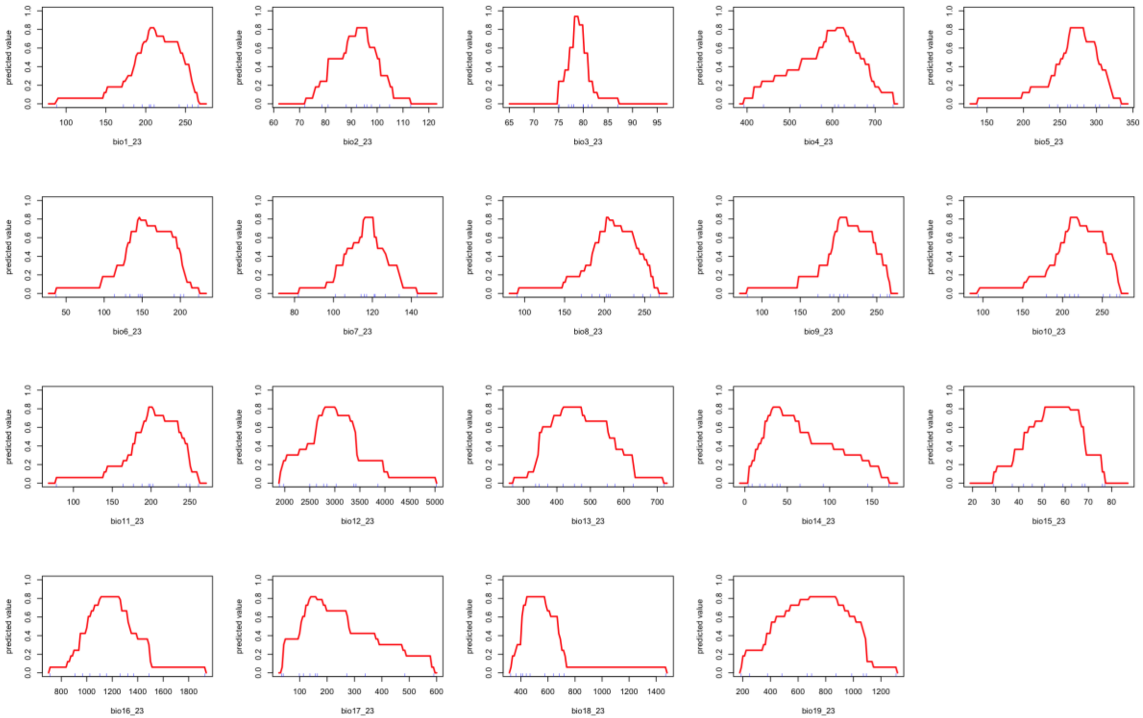


The second plot (Fig. 18) showed how the probability of the *Atelopus varius* occurrences (y-axes), from 0 to 1, differ with each bioclimatic predictors (x-axes). I focused on the 2 most common bioclimatic variables for Panama and Costa Rica, BIO1 (Mean Annual Temperature), and BIO12 (Mean Annual Precipitation). The bioclimatic temperature variables in BIO1 are shown in °C x 10.

BIO1 shows that below the threshold of about 6°C (43 °F) of temperature per year, the probability of the *Atelopus varius* occurrence was 0. The probability increased nonlinearly at about 17 °C (61 °F) per year, and the probability of this species occurrence at its peak at about 20.3 °C (68 °F) per year, is 0.8, then the probability of the *Atelopus varius* occurrence plateaued at 24 °C (75 °F) to 25 °C (77 °F), and decreased at 26 °C (79 °F) Therefore, the probability of this species to occur in its range was between 17 °C (61 °F) and 25 °C (77 °F) per year.

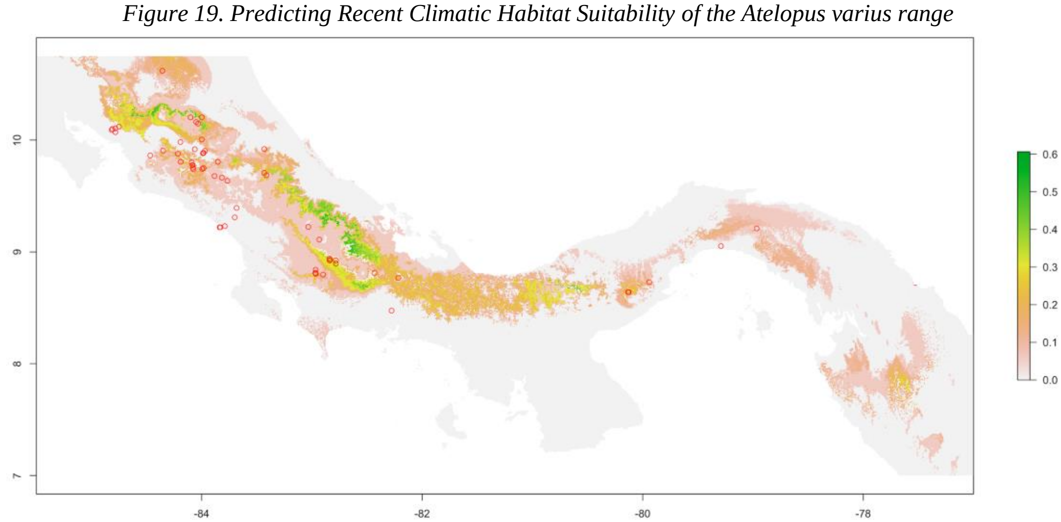
BIO12 showed the probability of the *Atelopus varius* occurrence below the threshold of about 1895 mm of precipitation per year is 0. The probability of this species occurrence increases nonlinearly at about 1916 mm of precipitation per year with 0.2 and reached its peak at 2785 mm to 3085 mm of precipitation per year with a probability of occurrence at 0.8, then the probability of the *Atelopus varius* to occur decreased nonlinearly at about 3426 mm per year. Therefore, the probability of the *Atelopus varius* to occur in its range is between 1916 mm and 3426 mm of precipitation per year.

Figure 18. Atelopus varius Response to Bioclimatic variables range of values based on the estimates of the probability of occurrences.



The habitat suitability plot (Fig. 19) predicted mostly areas of low suitability for *Atelopus varius*. From the 62 species observations, there were also 95% had areas with habitats where the probability of occurrence predicted to be below 0.2, which were not suitable for the *Atelopus varius* to survive.

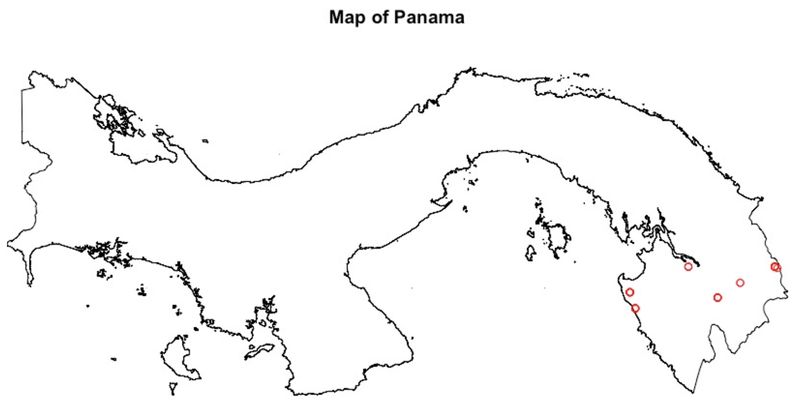
Figure 19. Predicting Recent Climatic Habitat Suitability of the Atelopus varius range



4.3.3 Darien Stubfoot Toad *(Atelopus certus)*

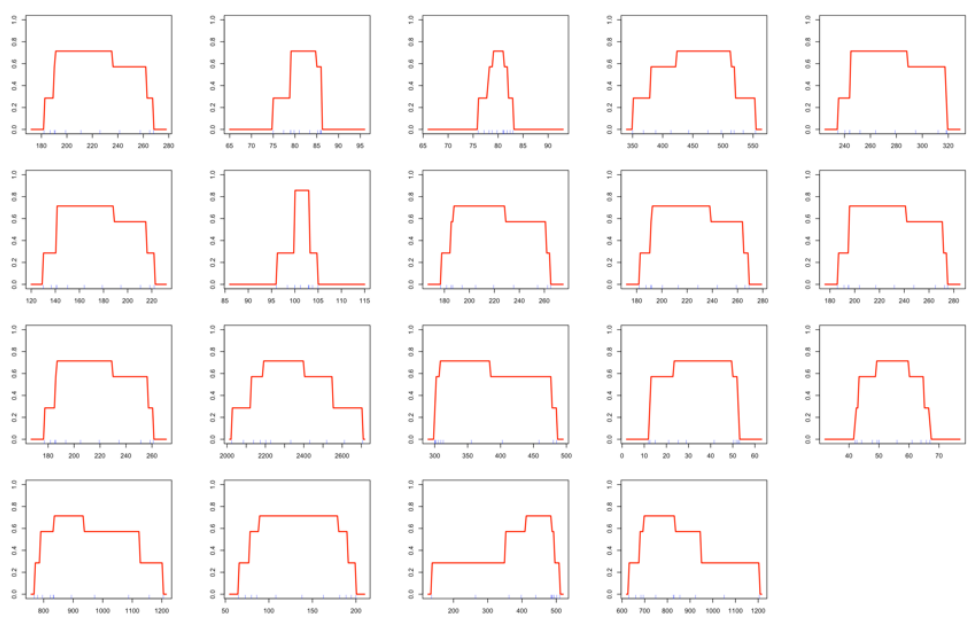
This is the preliminary map (Fig. 20) of the Darien Stubfoot Toad *(Atelopus certus)* distribution with biased observation points to see how this species was distributed in Darien National Park, East Panama at a mean elevation of 600 m.

Figure 20. Map of the Atelopus certus Distribution in Panama.



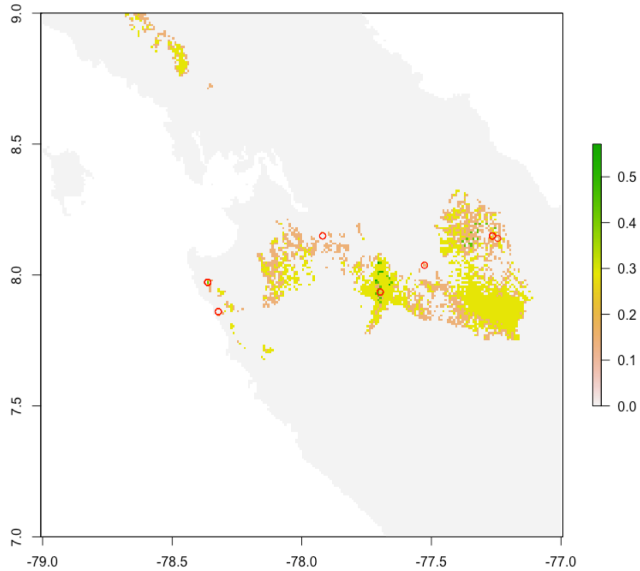
The response plot (Fig. 21) showed how the probability of the *Atelopus certus* occurrences (y-axes), from 0 to 1, differ with each bioclimatic predictors (x-axes). BIO1 showed that below the threshold of about 18°C (64 °F) of temperature per year, the probability of the *Atelopus certus* occurrence is 0. The probability increased linearly at about 18.5 °C (65 °F) per year, and the probability of this species occurrence at its peak at about 19 °C (66 °F) per year, is 0.6, then the probability of the *Atelopus certus* occurrence continued to increase up to 24 °C (74 °F) with a predicted value of 0.6, and the probability of this species occurrence decreased at about 26 °C (79 °F) Therefore, the probability of the *Atelopus certus* to occur in this range at a predicted value of 0.6 is between 18.5 °C (65 °F) and 26 °C (79 °F) per year. BIO12 showed the probability of the *Atelopus certus* occurrence below the threshold of about 2000 mm of precipitation per year with a predicted value of 0. The probability of this species occurrence increases nonlinearly at about 2100 mm of precipitation per year with a predicted value 0.3 and reached its peak at 2200 mm of precipitation per year with a probability of occurrence at 0.7, then decreases nonlinearly at about 2600 mm per year with a probability of occurrence of 0.2. Therefore, the probability of the *Atelopus certus* to occur in its range was between 2100 mm and 2600 mm of precipitation per year.

Figure 21. Figure 28. Atelopus certus Response to Bioclimatic variables range of values based on the estimates of the probability of occurrences.



What the habitat suitability plot (Fig. 22) predicted about the *Atelopus certus* distribution of suitable habitats in East Panama was that 10% of this species observed had suitable habitats predicted to be below 0.3, which were not suitable, and 90% of *Atelopus certus* observed in places where the probability of occurrence was predicted to be below 0.2, which were not suitable for the *Atelopus certus* to survive.

Figure 22. Predicting Recent Bioclimatic Habitat Suitability of the Atelopus certus range.



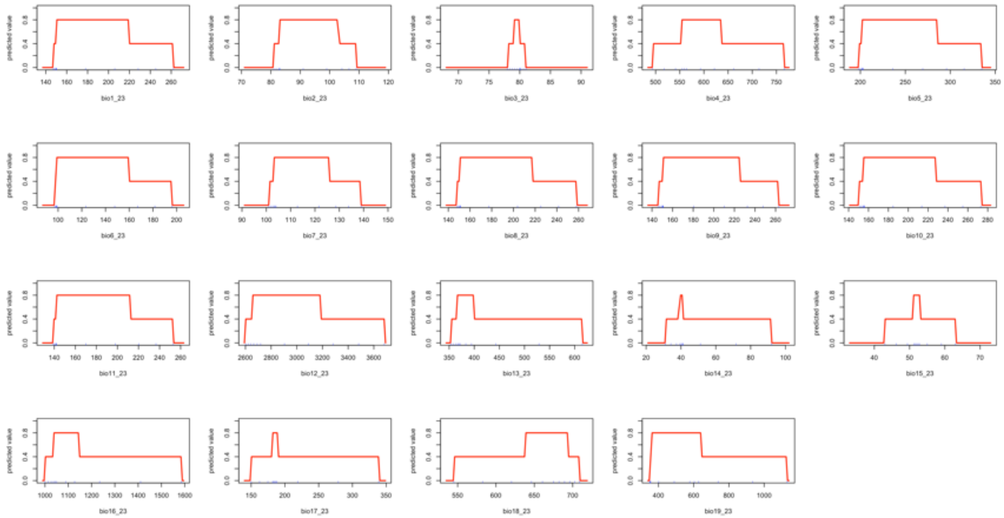
4.3.4 Pass Stubfoot Toad *(Atelopus senex)*

This is the preliminary map (Fig. 23) of the Pass Stubfoot Toad *(Atelopus senex)* distribution with biased observation points to see how this species was distributed in the Central Valley and Talamanca Mountain Range in Costa Rica from at a mean elevation of 1,650 meters.

Figure 23. Map of the Atelopus senex Distribution in Costa Rica

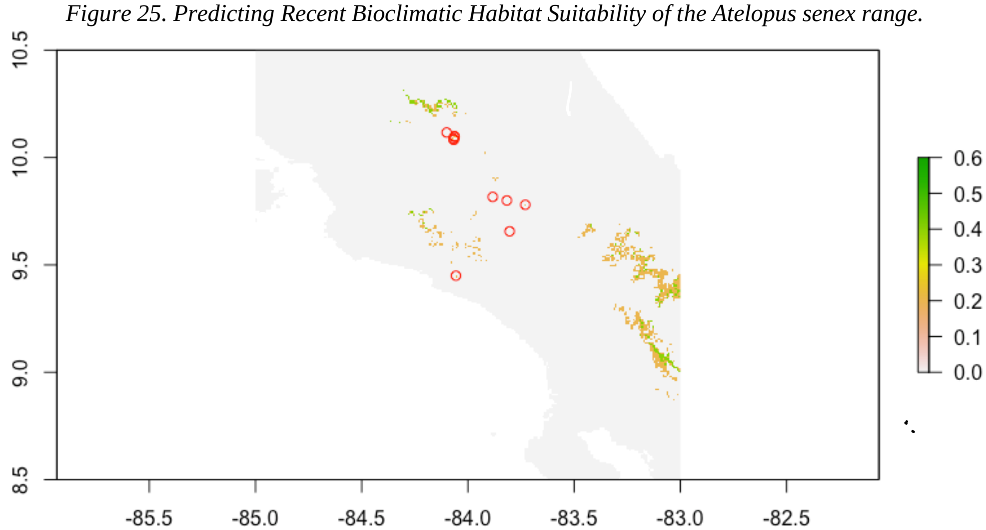
The response plot (Fig. 24) showed how the probability of the *Atelopus senex* occurrences (y-axes), from 0 to 1, differ with each bioclimatic predictors (x-axes). BIO1 showed that below the threshold of about 14°C (57 °F) of temperature per year, the probability of the *Atelopus senex* occurrence is 0. The probability of this species occurrence increased linearly and peaked at about 15 °C (59 °F) per year, with a predicted value of 0.8. The probability of the *Atelopus senex* occurrence plateaued from 15 °C (59 °F) to 22 °C (72 °F) with a predicted value of 0.8, and the probability of this species occurrence decreased at about 26 °C (79 °F) Therefore, the probability of this species to occur in this range at a predicted value of 0.6 is between 15 °C (59 °F) and 26 °C (79 °F) per year. BIO12 showed the probability of the *Atelopus senex* occurrence below the threshold of about 2600 mm of precipitation per year with a predicted value of 0. The probability of this species occurrence increases nonlinearly at about 2680 mm of precipitation per year with a predicted value 0.4 and reached its peak at about 2700 mm of precipitation per year with a probability of occurrence at 0.8. The probability of this species occurrence decreases nonlinearly at about 3700 mm per year with a probability of occurrence of 0.4. Therefore, the probability of the *Atelopus senex* to occur in its range was between 2680 mm and 3700 mm of precipitation per year.

Figure 24. Atelopus senex Response to Bioclimatic variables range of values based on the estimates of the probability of occurrences.



What the habitat suitability plot (Fig. 25) predicted about the *Atelopus senex* distribution of suitable habitats in Costa Rica was that 0% of this species observed had suitable habitats, and all of *Atelopus senex* observed in places where the probability of occurrence was predicted to be below 0, which were not suitable for the *Atelopus certus* to survive.

Figure 25. Predicting Recent Bioclimatic Habitat Suitability of the Atelopus senex range.



Finally, presented in Table 7, is a summary of the probability of occurrences of the Atelopus species in Panama and Costa Rica and how these amphibians responded to bioclimatic variables, temperature and precipitation, elevation, and habitat suitability in connection with species population declines and extinctions.

Table 7. Summary of the Atelopus species' Probability of Occurrence in BIO1 and BIO12, and the Probability of Occurrence with Habitat Suitability

***Atelopus* species**	**Country**	**Mean Elev. m**	**BIO1 °C**	**BIO1 Mean Temp °C**	**BIO1 Prob of occur**	**BIO12 mm**	**BIO12 Mean Precip**	**BIO12 Prob of occur**	**Hab Suitab below 0.3**	**Hab Suitab 0.0**
A. senex	Costa Rica	2000	15-26	21	0.6	2680-3700	3190	0.4	-	100%
A. chiriquiensis	Costa Rica Panama	1750	14-18	16	0.8	1760-3120	2440	1	70%	-
A. varius	Costa Rica Panama	1223	17-25	21	0.8	1916-3426	2671	0.8	100%	-
A. certus	Panama	600	18-26	22	0.6	2100-2600	2350	0.6	100%	-

CHAPTER 5 Discussion

5.1 Discussion

There are studies that have explored *Batrachochytrium dendrobatidis* (Bd) as the major factor that influenced the amphibian population decline in Central and South America. I found that there was a connection linked to the *Atelopus* species population decline influenced by Bd with their ecological characteristics consistent with past research (Lips, 2008; Crawford et al., 2010; La Marca et al., 2005). These included the *Atelopus* species life history, habitat type, home range and threats.

I noticed the *Atelopus* species that occurred at the maximum elevation first experienced extinction. The Costa Rican *Atelopus* species, *Atelopus chirripoensis*, *Atelopus senex*, *Atelopus chiriquiensis and Atelopus varius* occurred at mean elevation in descending order from 3450 m to 1750 m. However, the Panamanian *Atelopus* species did not match elevational pattern of extinction or population decline. The *Atelopus glyphus* of East Panama occurred at mean elevation of 1192 m and is still extant. The *Atelopus zeteki* of Central Panama occurred at a mean elevation of 825 m and this species is extinct in the wild. The persistent *Atelopus limosus* species occur in lower elevation sites at mean elevation of 370 m. This could indicate that the Bd spread and prevalence may have occurred in a geographical pattern consistent with prior studies from Lips et al., (2008). Although elevation has an influence in Bd spread, it is important to examine the different types of habitats and bioclimatic conditions the *Atelopus* species sustained in Panama and Costa Rica.

I noticed that the most common bioclimatic variables, temperature and precipitation, emphasized a significant relationship with Bd infection in the *Atelopus* species in Costa Rica and Panama. All *Atelopus* species, (except *Atelopus chirripoensis, Atelopus zeteki,* and *Atelopus glyphus*), occurred in sites where the mean temperature was between 16 ºC and 26 ºC, and the mean annual precipitation was between 1750 mm and 3190 mm. The habitats of all *Atelopus* species was not suitable for survival with a value under 0.3. These bioclimatic variables made the habitat of all *Atelopus* species suitable for Bd spread. These results are important because these factors could demonstrate the reason Bd is prevalent in the *Atelopus* species, which thrives in streams at high elevation montane forests but cannot survive temperatures at 28 ºC (82 ºF) in lowland forests (Gratwicke, 2019).

The new part of my research was the phylogenetic tree that highlighted the relationship between *Atelopus* species genetics and their extinction or population decline.

The phylogenetic tree split into 3 lineages, one of which developed into the *Atelopus glyphus,* then the second lineage developed into the *Atelopus limosus,* and the other which gave rise to *Atelopus varius* and *Atelopus chiriquiensis*. The *Atelopus varius* and *Atelopus chiriquiensis* share a more recent genetic similarities with each other than either shares with *Atelopus glyphus, Atelopus zeteki,* or *Atelopus limosus*. The *Atelopus varius* and the *Atelopus chiriquiensis* are therefore more closely related to each other than either is to *Atelopus glyphus, Atelopus zeteki* or *Atelopus limosus.* I noticed that the phylogenetic tree organized the species by genetic similarities, and also by geographical location of occurrence. For example, the *Atelopus chiriquiensis* and *Atelopus varius* both occurred in Costa Rica. The *Atelopus limosus* and *Atelopus zeteki* both occurred in Central Panama, and the *Atelopus glyphus* occurred in the Darien, East Panama. The *Atelopus varius, Atelopus limosus, Atelopus glyphus* are considered critically endangered by the IUCN (2019). The *Atelopus chiriquiensis* is considered extinct and the *Atelopus zeteki* is considered extinct in the wild. This finding means that these species are in serious threat of extinction. This finding also highlights that the *Atelopus* species are endemic, historically isolated and have less resistance to diseases and are significantly susceptible to Bd infection, consistent with Lips et al., (2008) hypothesis.

I learned that the decline in amphibian population has led to the extinction of endemic species resulting from Bd infections in connection with climate change. This means that bioclimatic factors, like temperature and precipitation has affected the outcome of Bd spread in the *Atelopus* species. Understanding the *Atelopus* species genetic diversity and immune defenses in connection to climate and environmental factors is important to find solutions to Bd spread in Central America and across the globe.

5.2 Limitations of the Study

The data to create forensic life history of the *Atelopus chirripoensis* from *Costa Rica*, *Atelopus glyphus,* and *Atelopus certus* from Panama using literature and information from the IUCN (2019) was deficient. There was only 1 single *Atelopus chirripoensis* observed in 1980 and no sample of this species was obtained. *Atelopus glyphus,* and *Atelopus certus* occurred in remote sites in Darién in Eastern Panama, where Colombian guerillas occupied. Therefore, the conservation status of these *Atelopus* will need further research.

I conducted a species distribution analysis using data of 8 *Atelopus* species using R and utilized only occurrence data with coordinates of location focused in Costa Rica and Panama, and bioclimatic variables. To obtain unbiased data for the habitat suitability model, occurrences at the same coordinates were removed. Because of the reduction of occurrences, the *Atelopus zeteki*, the *Atelopus limosus* and the *Atelopus glyphus* did not produce results. The *Atelopus chirripoensis* occurrence had only 1 observation and did not produce a result.

The data used to analyze the *Atelopus* genetic relationship with extinction using a phylogenetic tree did not include the DNA sequence data of the 8 *Atelopus* species for my research. There was no collection of the 16S ribosomal RNA sequence for the *Atelopus chirripoensis*, *Atelopus senex*, and *Atelopus certus* to produce a DNA sequence alignment. As a result, only 5 *Atelopus* species of Panama and Costa Rica were identified.

The methods used for this research included limitations. The analysis for species distribution model, I used R to produce a species distribution model to analyze climatically suitable habitats based only on historical bioclimatic variables from 1970 to 2000.

The main foundation of this thesis was based on existing data of amphibian decline and extinctions from La Marca et al. (2005), existing RNA sequence data of the *Atelopus* species from The National Center for Biotechnology Information (NCBI), and species distribution data from the International Union for Conservation of Nature's Red List of Threatened Species (IUCN). All peer-reviewed literature was collected from the Evergreen State College Online library.

5.4 Next Steps and Future Studies

Determining the influence historical bioclimatic variables had in connection with Bd prevalence in the *Atelopus* species was an important part of my thesis. The *Atelopus* species life history explained important ecological traits and genetic associations that could have impacted their population declines and extinctions.

To obtain accurate data of the *Atelopus* species conservation status, a new assessment, including the *Atelopus* species with low occurrence observations, needs to be implemented. In addition, there are many studies about Panamanian *Atelopus* species, but little is known about the extinctions of the Costa Rican *Atelopus* species.

I propose future studies that can address future suitable bioclimate conditions for the *Atelopus* species in the next 30 years to determine appropriate Bd mitigation plan and amphibian conservation and management for future reintroduction of these species.

5.5 Conclusion

Amphibians around the world have been experiencing habitat loss, ecological changes, anthropogenic impact, and disease that affect their population. However, the primary cause of amphibian population declines, and extinction is Chytridiomycosis, an amphibian skin infection caused by a fungus, *Batrachochytrium dendrobatidis* (Bd).

Amphibians are most at risk to becoming extinct. There have been many events that have led to amphibian decline, however climatic factors could have influenced Bd spread and infections. However, changes in their environment could impact their ability to survive.

A significant tendency for amphibians suggests a consistent decline in diversity. Costa Rica and Panama are well known for their rich biodiversity in Central America and host the most endangered vertebrate species, the Harlequin frogs of the *Atelopus* genus. Most *Atelopus* species occur in the upland rainforests where Bd prevalence is higher. Three Costa Rican *Atelopus* species have been considered extinct, and one Panamanian *Atelopus* species is extinct in the wild caused by Bd.

There have been many efforts to preserve the *Atelopus* species. Most of the *Atelopus* species in Costa Rica are now extinct without live specimens in captivity due to the unknown nature of their disappearance. Studies using an *Atelopus* species' skin bacteria to help reduce Bd infections have not been successful. However, one *Atelopus* species in Panama has shown persistence to Bd, indicating that there is an opportunity for further analyses to prevent Bd infections in other *Atelopus* species. Moreover, efforts to reintroduce the *Atelopus limosus* species into the wild in East Panama in 2017 did not identify any change in their survival rate. A captive conservation program for the *Atelopus* species by the Smithsonian Tropical Research Institute have been established to breed and ensure a sustainable population for reintroduction into the wild.

My thesis focused to determine the cause of Bd prevalence in the rainforests of Panama and Costa Rica, the effect Bd prevalence in the *Atelopus* species and the relationship of Bd with climate change. Understanding how Bd connects with climate in the rainforest of Central and South America can help prevent future amphibian extinctions. The effect of Bd on the *Atelopus* species could have been due to their genetic lineage and bioclimatic factors such as temperature and precipitation and high-altitude environment.

The *Atelopus* life history and phylogenetic tree data showed a connection to extinction and decline caused by Bd. The *Atelopus* species in Panama and Costa Rica shared common characteristics, which are the occurrences in aquatic habitats, and high elevational home ranges with topographic constraints that have influenced their population declines and extinctions.

The phylogenetic tree indicated that the *Atelopus* species are vulnerable because of their low genetic variation, as a consequence, the *Atelopus* species with close genetic relationship experienced extinction related to their endemic environment. This data supports the amphibian lineage and diversity loss hypothesis by Crawford, Lips and Bermingham (2010).

Although the *Atelopus* species had a drastic population decline and extinction, one species is persisting Bd infection in previously prevalent sites (Voyles et al., 2018). The recovery of this *Atelopus* species provides a useful tool for future scientific research to understand the effect of Bd and amphibian response to bioclimatic variables as a result of climate change, in the hope of finding a solution to the chytrid epidemic around the world. Consequently, a captive breeding program is in effect to ensure the survival of the *Atelopus* species with the purpose of reintroducing these amphibian species back to the wild.

Bibliography

Amphibian Chytrid Fungus. *Emerging Infectious Diseases*, *10*(12), 2100-2105. Retrieved from https://dx.doi.org/10.3201/eid1012.030804

Amphibian Red List Authority. (2017, November 17). Amphibian Survival Alliance. Retrieved from http://www.amphibians.org/redlist/

Baillie, J.E.M., Hilton-Taylor, C. and Stuart, S.N. (Editors) 2004. *2004 IUCN Red List of Threatened Species. A Global Species Assessment*. IUCN, Gland, Switzerland and Cambridge, UK. xxiv + 191 pp.

Becker, M., Harris, R., Minbiole, K., Schwantes, C., Rollins-Smith, L., Reinert, L., … Gratwicke, B. (2011). Towards a Better Understanding of the Use of Probiotics for Preventing Chytridiomycosis in Panamanian Golden Frogs. *EcoHealth*, *8*(4), 501–506. https://doi.org/10.1007/s10393-012-0743-0

Belden, L. K., Hughey, M. C., Rebollar, E. A., Umile, T. P., Loftus, S. C., Burzynski, E. A., … Harris, R. N. (2015). Panamanian frog species host unique skin bacterial communities. *Frontiers in Microbiology, 6*. https://doi.org/10.3389/fmicb.2015.01171

Blaustein, A. R., Han, B. A., Relyea, R. A., Johnson, P. T. J., Buck, J. C., Gervasi, S. S., & Kats, L. B. (2011). The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. *Annals of the New York Academy of Sciences*, *1223*(1), 108–119. https://doi.org/10.1111/j.1749-6632.2010.05909.x

Bolaños, F., Chaves, G. & Barrantes, U. 2008. *Atelopus senex*. *The IUCN Red List of Threatened Species* 2008:

e.T54549A11165586. https://dx.doi.org/10.2305/IUCN.UK.2008.RLTS.T54549A11165586.en.

Brucker RM, Harris RN, Schwantes CR, Gallaher TN, Flaherty DC, Lam BA, Minbiole KPC. (2008). Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander Plethodon cinereus. J Chem Ecol 34: 1422–1429

Collins, J. P., Crump, M. L., & Lovejoy, T. E. I. (2009). *Extinction in our times: Global amphibian decline*. Retrieved from https://ebookcentral-proquest-com.evergreen.idm.oclc.org

Colón-Gaud, C., Whiles, M. R., Lips, K. R., Pringle, C. M., Kilham, S. S., Connelly, S., … Peterson, S. D. (2010). Stream invertebrate responses to a catastrophic decline in consumer diversity. *Journal of the North American Benthological Society*, *29*(4), 1185–1198. https://doi.org/10.1899/09-102.1

Cochran, D. M., & Goin, C. J. (1970). Frogs of Colombia. *Bulletin of the United States National Museum*, (288), 1–655. https://doi.org/10.5479/si.03629236.288.1

Costa Rica to Panama: Stephen Fry in Central America [Video file]. (2015). Retrieved May 11, 2020, from https://fod.infobase.com/PortalPlaylists.aspx?wID=107687&xtid=115681

Crawford, A., Lips, K., Bermingham, E., & Wake, D. (2010). Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proceedings of the National Academy of Sciences of the United States of America, 107*(31), 13777-13782. Retrieved from http://www.jstor.org.evergreen.idm.oclc.org/stable/25708801

Cheng, T. L., Rovito, S. M., Wake, D. B., and Vredenburg, V. T. (2011). Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. Proceedings of the National Academy of Sciences USA. Published online before print May 4, 2011, doi: 10.1073/pnas.1105538108.

Definition of NEOTROPICAL. (n.d.). Retrieved March 1, 2019, from https://www.merriam-webster.com/dictionary/neotropical

Dodd Jr., C. K. (2009). *Amphibian Ecology and Conservation : A Handbook of Techniques*. Retrieved from http://ebookcentral.proquest.com/lib/esu/detail.action?docID=1657783

Dunn, E. R. (1931). "New frogs from Panama and Costa Rica." *Occasional Papers of the Boston Society of Natural History*, 5, 385-401.

Ellison, A. R., Tunstall, T., DiRenzo, G. V, Hughey, M. C., Rebollar, E. A., Belden, L. K., … Zamudio, K. R. (2015). More than skin deep: functional genomic basis for resistance to amphibian chytridiomycosis. *Genome Biology and Evolution*, *7*(1), 286–298. https://doi.org/10.1093/gbe/evu285

First Release Trial (2017, June). *Panama Amphibian Rescue and Conservation Project*. Retrieved from http://amphibianrescue.org/2017/06/01/first-release-trial-to-help-pave-the-way-for-reintroduction-programs-for-critically-endangered-frogs/

Fick, S.E. and R.J. Hijmans, 2017. WorldClim 2: new 1km spatial resolution climate surfaces for global land areas. International Journal of Climatology 37 (12): 4302-4315.

Flechas, S. V., Sarmiento, C., Cárdenas, M. E., Medina, E. M., Restrepo, S., Amézquita, A. (2012). "Surviving chytridiomycosis: Differential anti-*Batrachochytrium dendrobatidis* activity in bacteria isolates from three lowland species of *Atelopus*." *PLOS ONE*, 7(9).

Flechas, S. V., Blasco-Zúñiga, A., Merino-Viteri, A., Ramírez-Castañeda, V., Rivera, M., & Amézquita, A. (2017). The effect of captivity on the skin microbial symbionts in three Atelopus species from the lowlands of Colombia and Ecuador. *PeerJ*, *2017*(7), e3594. https://doi.org/10.7717/peerj.3594

González-Maya, J. F., Belant, J. L., Wyatt, S. A., Schipper, J., Cardenal, J., Escobedo-Galván, A. H., … Corrales, D. (2013). Renewing hope: The rediscovery of Atelopus varius in Costa Rica. *Amphibia Reptilia*, *34*(4), 573–578. https://doi.org/10.1163/15685381-00002910

González-Maya, J. F., Gómez-Hoyos, D. A., Cruz-Lizano, I., & Schipper, J. (2018). From hope to alert: demography of a remnant population of the Critically Endangered Atelopus varius from Costa Rica. *Studies on Neotropical Fauna and Environment*, *53*(3), 194–200. https://doi.org/10.1080/01650521.2018.1460931

Gratwicke, B., Ross, H., Batista, A., Chaves, G., Crawford, A. J., Elizondo, L., … Ibáñez, R. (2016). Evaluating the probability of avoiding disease-related extinctions of Panamanian amphibians through captive breeding programs. *Animal Conservation*, *19*(4), 324–336. https://doi.org/10.1111/acv.12249

Groom, M., Meffe, G. K. & Carroll, C. R. (2006). *Principles of conservation biology*. 3rd edn. Sinauer Associates, Sunderland, MA.

Halliday, T. R. (2008, July). Why amphibians are important. *International Zoo Yearbook*, Vol. 42, pp. 7–14. https://doi.org/10.1111/j.1748-1090.2007.00037.x

Hayes, M. P., Rombough, C. J., Padgett-flohr, G. E., Lisa, A., Johnson, J. E., Wagner, R. S., … Engler, J. D. (2009). *Amphibian Chytridiomycosis in the Oregon Spotted Frog (Rana pretiosa) in Washington Published by : Society for Northwestern Vertebrate Biology Stable URL : https://www.jstor.org/stable/20628128 REFERENCES Linked references are available on JSTOR for thi*. *90*(2), 148–151.

Ibañez, R., Jaramillo C., & Solis F. (1995). Another species of Atelopus (Amphibia: Bufonidae) of Panama. *Caribbean Journal of Science,* 31(1), 57-64.

Ibañez, R, AS Rand, and CA Jaramillo. (1999). Los anfibios del monumento natural barro colorado, parque nacional soberanía y areas adyacentes. Mizrachi, E. and Pujol, S.A., Santa Fe de Bogota.

Ibáñez, R., Solís, F., Jaramillo, C., Fuenmayor, Q., Lötters, S, Rueda, J. V., and Acosta-Galvis, A. (2004). *Atelopus glyphus*. In: IUCN 2008. 2008 IUCN Red List of Threatened Species. www.iucnredlist.org.

Ibáñez, R. (2015). *Atelopus glyphus*. In: IUCN 2019. 2019 IUCN Red List of Threatened Species. www.iucnredlist.org

Ibáñez, R. (2018). *Atelopus glyphus*. In: IUCN 2019. 2019 IUCN Red List of Threatened Species. www.iucnredlist.org

IUCN SSC Amphibian Specialist Group & NatureServe. 2013. *Atelopus chirripoensis*. *The IUCN Red List of Threatened Species* 2013: e.T186064A1809697. https://dx.doi.org/10.2305/IUCN.UK.2013-1.RLTS.T186064A1809697.en.

IUCN SSC Amphibian Specialist Group. 2019. *Atelopus certus*. *The IUCN Red List of Threatened Species* 2019: e.T54497A54340637. https://dx.doi.org/10.2305/IUCN.UK.2019-3.RLTS.T54497A54340637.en.

IUCN SSC Amphibian Specialist Group. 2019. *Atelopus glyphus*. *The IUCN Red List of Threatened Species* 2019: e.T54514A49535891. https://dx.doi.org/10.2305/IUCN.UK.2019-3.RLTS.T54514A49535891.en.

IUCN SSC Amphibian Specialist Group. 2019. *Atelopus limosus*. *The IUCN Red List of Threatened Species* 2019: e.T54520A54340943. https://dx.doi.org/10.2305/IUCN.UK.2019-3.RLTS.T54520A54340943.en.

IUCN SSC Amphibian Specialist Group. 2019. *Atelopus zeteki*. *The IUCN Red List of Threatened Species* 2019: e.T54563A54341110. https://dx.doi.org/10.2305/IUCN.UK.2019-3.RLTS.T54563A54341110.en.

Johnson, M.L. and Speare, R. (2005). Possible modes of dissemination of the amphibian chytrid Batrachochytrium dendrobatidis in the environment. *Dis. Aquatic*. Org. 65, 181–186.

Kilpatrick, A. M., Briggs, C. J., and Daszak, P. (2010). The ecology and impact of Chytridiomycosis: an emerging disease of amphibians. *Trends in Ecology & Evolution*, *25*(2), 109–118. https://doi.org/10.1016/j.tree.2009.07.011

Kirshtein, J. D., Anderson, C. W., Wood, J. S., Longcore, J. E., & Voytek, M. A. (2007). Quantitative PCR detection of Batrachochytrium dendrobatidis DNA from sediments and water. *Diseases of Aquatic Organisms*, *77*(1), 11–15. https://doi.org/10.3354/dao01831

Kolbert, E. (2014). *The sixth extinction : An Unnatural history* (First ed.). New York: Henry Holt and Company.

La Marca, E., Lips, K. R., Lötters, S., Puschendorf, R., Ibáñez, R., Rueda-Almonacid, J. V., … Young, B. E. (2005). Catastrophic population declines and extinctions in neotropical harlequin frogs (Bufonidae: Atelopus). *Biotropica*, Vol. 37, pp. 190–201. https://doi.org/10.1111/j.1744-7429.2005.00026.x

Lewis, C. H. R., Richards-Zawacki, C. L., Ibáñez, R., Luedtke, J., Voyles, J., Houser, P., & Gratwicke, B. (2019). Conserving Panamanian harlequin frogs by integrating captive-breeding and research programs. *Biological Conservation*, *236*, 180–187. https://doi.org/10.1016/j.biocon.2019.05.029

Lips, K. R., Reeve, J. D., & Witters, L. R. (2003). Ecological Traits Predicting Amphibian Population Declines in Central America. *Conservation Biology*, *17*(4), 1078–1088. https://doi.org/10.1046/j.1523-1739.2003.01623.x

Lips, K. R., Diffendorfer, J., Mendelson, J. R., & Sears, M. W. (2008). Riding the Wave: Reconciling the Roles of Disease and Climate Change in Amphibian Declines. PLoS Biology, 6(3), e72. https://doi-org.evergreen.idm.oclc.org/10.1371/journal.pbio.0060072

Lips, K., Ibáñez, R., Bolaños, F., Chaves, G., Solís, F., Savage, J., Jaramillo, C., Fuenmayor, Q. & Castillo, A. 2010. *Atelopus chiriquiensis*. *The IUCN Red List of*

Threatened Species 2010: e.T54498A11144381. https://dx.doi.org/10.2305/IUCN.UK.2010-2.RLTS.T54498A11144381.en.

Longcore et al. (1999). Infection of frogs by amphibian chytrid causing the disease chytridiomycosis. Retrieved from http://www.environment.nsw.gov.au/research-and-publications/publications-search/infection-of-frogs-by-amphibian-chytrid-causing-the-disease-chytridiomycosis

Markle, S. (2012). *The case of the vanishing golden frogs : A scientific mystery*. Minneapolis: Milbrook Press.

Noss, R. F. & Cooperrider, A. Y. (1994). *Saving and restoring biodiversity.* Island Press, Washington D.C.

O'Hanlon, S. J., Rieux, A., Farrer, R. A., Rosa, G. M., Waldman, B., Bataille, A., Fisher, M. C. (2018). Recent Asian origin of chytrid fungi causing global amphibian declines. *Science*, *360*(6389), 621–627. https://doi.org/10.1126/science.aar1965

Pounds, J. A., & Crump, M. L. (1994). Amphibian Declines and Climate Disturbance: The Case of the Golden Toad and the Harlequin Frog. *Conservation Biology*, *8*(1), 72–85. https://doi.org/10.1046/j.1523-1739.1994.08010072.x

Pounds, J., Puschendorf, R., Bolaños, F., Chaves, G., Crump, M., Solís, F., Ibáñez, R., Savage, J., Jaramillo, C., Fuenmayor, Q. & Lips, K. 2010. *Atelopus varius*. *The IUCN Red List of Threatened Species* 2010: e.T54560A11167883. https://dx.doi.org/10.2305/IUCN.UK.2010-2.RLTS.T54560A11167883.en.

R Core Team (2020). R: A language and environment for statistical computing. R

Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Richards-Zawacki, C. L. (2009). Effects of slope and riparian habitat connectivity on gene flow in an endangered panamanian frog, atelopus varius. *Diversity and Distributions*, *15*(5), 796–806. https://doi.org/10.1111/j.1472-4642.2009.00582.x

Rodríguez-Brenes, S., Rodriguez D., Ibáñez, R., & Ryan, M. J. (2016). Spread of Amphibian Chytrid Fungus across Lowland Populations of Túngara Frogs in Panamá. *PLoS ONE*, *11*(5), e0155745. https://doi.org/10.1371/journal.pone.0155745

Rosenblum, E., Stajich, J., Maddox, N., & Eisen, M. (2008). Global gene expression profiles for life stages of the deadly amphibian pathogen Batrachochytrium dendrobatidis. Proceedings of the National Academy of Sciences of the United States of America, 105(44), 17034-17039.

Rosenblum, E. B., Voyles, J., Poorten, T. J., & Stajich, J. E. (2010). The Deadly Chytrid Fungus: A Story of an Emerging Pathogen. *PLOS Pathogens*, *6*(1), e1000550. https://doi.org/10.1371/journal.ppat.1000550

Ryan, M. J., Berlin, E., Gagliardo, R. W., & Lacovelli, C. (2005). Further exploration in search of Atelopus varius in CostaRica. *Froglog*, *69*(January 2005), 1–2.

Stuart, S., Hoffmann, M., Chanson, J., Cox, N., Berridge, R., Ramani, P., Young, B. (eds) (2008). *Threatened Amphibians of the World.* Lynx Edicions, IUCN, and Conservation International, Barcelona, Spain; Gland, Switzerland; and Arlington, Virginia, USA.

Valencia-Aguilar, A., Cortés-Gómez, A. M., & Ruiz-Agudelo, C. A. (2013). Ecosystem services provided by amphibians and reptiles in Neotropical ecosystems. *International Journal of Biodiversity Science, Ecosystem Services & Management*, *9*(3), 257–272. https://doi.org/10.1080/21513732.2013.821168

Vo, P., & Gridi-Papp, M. (2017). Low temperature tolerance, cold hardening and acclimation in tadpoles of the neotropical túngara frog (Engystomops pustulosus). *Journal of Thermal Biology*, *66*, 49–55. https://doi.org/10.1016/j.jtherbio.2017.03.012

Walker, S. F., Salas, M. B., Jenkins, D., Garner, T. W. J., Cunningham, A. A., Hyatt, A. D., … Fisher, M. C. (2007). Environmental detection of Batrachochytrium dendrobatidis in a temperate climate. *Diseases of Aquatic Organisms*, *77*(2), 105–112. https://doi.org/10.3354/dao01850

Weldon, C., du Preez, L. H., Hyatt, A. D., Muller, R., & Speare, R. (2004, December 12). Origin of the Amphibian Chytrid Fungus *Emerging Infectious Disease journal* - CDC. https://doi.org/10.3201/eid1012.030804

What Stops Mass Extinctions. (2018). Panama Amphibian Rescue and Conservation Center, Gamboa, Panama. Retrieved from https://stri.si.edu/story/what-stops-mass-extinctions

Wilson, D. (2014). "Frog Friday: Limosa Harlequin Frog. Panama Amphibian Rescue and Conservation Project." http://amphibianrescue.org/2014/10/03/atelopuslimosus/.

Young et al. (2001). Population Declines and Priorities for Amphibian Conservation in Latin America. *Conservation Biology,15*(5), 1213-1223. Retrieved from http://www.jstor.org.evergreen.idm.oclc.org/stable/3061476

Zippel, K. (2002). Conserving the Panamanian golden frog: Proyecto rana dorada. *Herpetological Review, 33*(1), 11-12.

Zippel, K. C., Ibáñez, R., Lindquist, E. D., Richards, C. L., Jaramillo, C. A., & Griffith, E. J. (2006). *Implicaciones en la conservación de las ranas doradas de Panamá, asociadas con su revisión taxonómica*. Retrieved from http://repository.si.edu/xmlui/handle/10088/12256