Ecology and Conservation Genetics of Mesoamerican Amphibians

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PhD 2020

Ecology and Conservation Genetics of Mesoamerican Amphibians

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A thesis submitted in partial fulfilment of the requirements of the Manchester Metropolitan University for the degree of Doctor of Philosophy

School of Science and the Environment
Faculty of Science and Engineering
Manchester Metropolitan University

2020

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General abstract

Mesoamerica is a biodiversity hotspot harbouring a great diversity of ecosystems and species. Amphibians are one of the most diverse groups in the region, unfortunately many populations are declining, and a considerable number have gone extinct. Amphibian populations are a fundamental and irreplaceable part of the ecosystems present in Mesoamerica and their loss would be devastating for Central American biodiversity therefore, understanding the pressures these populations are under and conserving them more effectively is of the upmost importance.

Molecular genetics is a useful tool to understand more about a population's diversity and its interactions with its environment. For the first part of this project I developed microsatellite markers for the black-eyed tree frog *Agalychnis moreletii* to study the genetic diversity and population structure of the species. Then I used mitochondrial markers to study the genetic and evolutionary history of the species. I found that several populations have low genetic diversity as well as high inbreeding levels. There is genetic differentiation and population structure across its distribution range. Four different Evolutionary Significant Units (ESUs) were identified for *A. moreletii*, and it is crucial to create different management plans for each one since there are different pressures threatening them. We also believe this data challenges the conservation status of the *A. moreletii* as it is currently defined by the IUCN and shows this species is far more endangered than currently thought. Changing this categorisation will increase the protection of the species and the ecosystem it inhabits.

I characterised the skin-bacterial communities of *A. moreletii* in Guatemalan populations and how the presence of the pathogen *Batrachochytrium dendrobatidis* has an effect on the bacterial composition. Skin-microbiome in *A. moreletii* seems host-mediated. Infected frogs harbour lower diversity of bacteria that non-infected frogs, which leaves these individuals more vulnerable.

For the final part of the PhD I studied the skin-bacterial composition of *Bolitoglossa* salamanders and *Plectrohyla matudai* and how it changes once the animals are taken from the wild into captivity. I documented that the bacterial diversity and composition declines when animals are in quarantine, which can be a stressful stage of a captive study since it usually involves a small container with paper towels. This shows that tank enrichment is important in maintaining a diverse skin-microbiome in captive amphibians and therefore tank diversity is crucial in maintaining the health of individuals.

The data gathered for my PhD will contribute to the conservation of amphibians and their ecosystems in Mesoamerica.

Dedication

To my dad, Gustavo Zamora Hernández.

Thanks for everything, I love you.

Acknowledgments

I would like to thank Professor Richard Preziosi for the opportunity to do my PhD at his laboratory, for all the guidance and help through difficult times. To all the members of the Ecological Genetics and Conservation Lab. Dr. Jenny Rowntree for all the support, Xaali, Diana, Jade, Guuske, Calum, Sarah for all the good times and friendship. Mariana and Miguel thanks for all the fun moments and the climbing sessions. Selvino De Kort and Ellie McLaren for all the help with the acoustics analysis. Liliana Solano for providing samples and always been an amazing friend.

To my amazing family for supporting my wildest dreams! A huge thanks to my parents, who sacrificed so much to give us the best education possible. Thanks for all the love and for encouraging me to see the world. To Luis for always being my compa, love you girl! Nela, Luna, Félix and Oso thanks for the stress relief sessions.

Thomas for all the laughs, great adventures, love and patience along this path, you are my rock! To my British family, the Hughes thanks for all the support and love.

This project would not have been without the support of countless people, but I would like to say a special thank you to the following. To the Biology department at Universidad del Valle de Guatemala, especially to Gabriela Alfaro and Priscila Juárez. The husbandry project would not have been possible without the hard work of Daniel Gygax, thank you very much! Erick López, thanks for always helping me with my projects and being a great friend. Luis Carrillo and Amphibian Ark for providing training and assistance for the husbandry facilities. Field work and sampling collection in Guatemala were achieved thanks to the hard work of Thomas, Dani, Liz, Rodrigo, Sofía, Jenny, Andrés. As well as the owners, managers and guides from Finca Los Tarrales, Finca Los Andres, Finca Las Nubes, especial thanks to Don Max, Finca El Patrocinio and to Don Paulino Chuvac. Finca Dulce Rocío and La Marsella and to the García Family for all the hospitality. Finca Sach-Wa Ja and Marcelo Serrano and Finca La Gracia and Martín Viteri.

Special thanks to Raquel Alvarado and Licda. Eunice Echeverría from the National History Museum at UES. Field work in El Salvador was successful thanks to Raquel Alvarado, Néstor Ruballo, Jaime Mejía, Melvin Salinas, the Staff at Los Andres, Volcán Santa Ana, Don Roberto, Don Amadeo, Dain Abizai, Leonel and Don Andrés Hernández. Don Manuel from Buenos Aires thank you for taking care of the frogs and opening your house for us.

Honduras field trips were possible with the assistance of Julio Mérida, Profe Cruz, Anthonie Mazariegos and Ninfa Mejía of Museo de Historia Natural at UNAH, Mario Solis, Enzo Carias, Jagoba Malumbres, Alejandra López and María Fernanda Sandoval. In Belize we were assisted by Friends of Nature Belize and all the personal from Las Cuevas Research Station. Mexico field work was done thanks to Luis Antonio Muñoz and Luis Caloca from ECOSUR, thanks for all the guidance and efforts. Permits were provided by Luis Muñoz and Omar Hernández at UNAM.

Fundación Guatefuturo provided a credit-scholarship to fund part of my PhD. Manchester Metropolitan University granted me a scholarship. To Consejo Nacional de Áreas Protegida (CONAP) in Guatemala, Ministerio de Ambiente y Recursos Naturales (MARN) in El Salvador, Forrestry Department in Belize and Secretaria de Recursos Naturales y Ambiente in Honduras for the issue of collection and exportation permits.

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Supplementary information - Chapter 5

Chapter 1 - General Introduction

Mesoamerica, a biodiversity hotspot

A biodiversity hotspot is defined as a biogeographic region with more than 1,500 endemic vascular plant species, that has lost 70% or more of its primary vegetation (Myers *et al.*, 2000). There are 35 biodiversity hotspots containing approximately 50% of the world's endemic plants and 42% of terrestrial vertebrates (Mittermeier *et al.*, 2011; Marchese, 2015). Biodiversity hotspots are mainly found in tropical forests covering only 2.3% of the Earth's land surface (Mittermeier *et al.* 2011) providing 35% of the global ecosystem services (Conservation International 2014).

Mesoamerica is a biodiversity hotspot that covers 1,130,019 km^2 of the Neotropical region and includes a wide variety of ecosystems, making it one of the most diverse and rich regions in the world (Myers *et al.* 2000; Muñoz & Mondini, 2008). It includes all the subtropical and tropical ecosystems from central Mexico and Central America up to the Panama Canal. In the area, there are 17 endemic bird areas, approximately 440 species of mammals (15% endemic), 690 species of reptiles (35% endemic), more than 550 species of amphibians (64% endemic) and at least 500 species of fish (Conservation International 2018).

The biological resources in this area are being threatened by population growth, economic inequality and underdevelopment. A lot of the protected areas in Mesoamerica are endangered due to unsustainable logging, the expansion of agricultural land, illegal traffic of flora and fauna, and infrastructure development (DeFries *et al.* 2005; Conservation International 2018). Remaining natural areas consist of small parks or reserves with fragmented forests (Miller *et al.* 2001) or forests surrounded by local communities that utilize the natural resources for their subsistence (DeFries *et al.*, 2005).

To understand why Mesoamerica has a high degree of biodiversity and endemism, several events have to be taken into consideration, the most relevant events are: the Chicxulub crater, resulting from a meteorite impact in the Yucatán Peninsula 64.5 million years ago (Mya); the formation and complete closure of Central American Isthmus; two glacial periods during the Pleistocene (Schuster & Bonis, 2008).

Temperature changes after the last glacial period during the Pleistocene have contributed to the high levels of diversity in the tropics. During this time the tropical forests were not as extensive as they are now due to the lower temperatures (Pleistocene Refugia Hypothesis) (Haffer 1969). At the end of the glacial period, around 11,600 years ago, the temperatures and humidity levels rose again allowing the remaining rainforest patches to expand and form continuous forests again (Haffer 1969).

After the glacial periods the cloud forests migrated to higher altitudes, influenced by the rise of temperature and re-colonization of the rainforests. As a consequence, the connectivity of these forests was lost, and the isolated populations evolved independently. This also explains the high numbers of endemic species in the area [e.g. insects (Schuster y Cano 2005; Schuster y Cano 2006), mammals (León-Paniagua *et al.*, 2007; López-González & García-Mendoza, 2012; García-Mendoza & López-González, 2013), reptiles and amphibians (Stuart & Arbor, 1943; Castoe *et al.*, 2009; Mata-Silva *et al.*, 2019)]. The high levels of endemism are also a result of several geographic barriers between Mexico and Central America (Figure 1). These includes the Mexican highlands at the southwest of the country, the Tehuantepec Isthmus, the Chiapan-Guatemalan highlands or Sierra Madre del Sur, the Motagua-Polochic fault, the highlands of the Chortís block, the Nicaraguan depression and the highlands between Costa Rica and Panamá (Castoe *et al.* 2009).

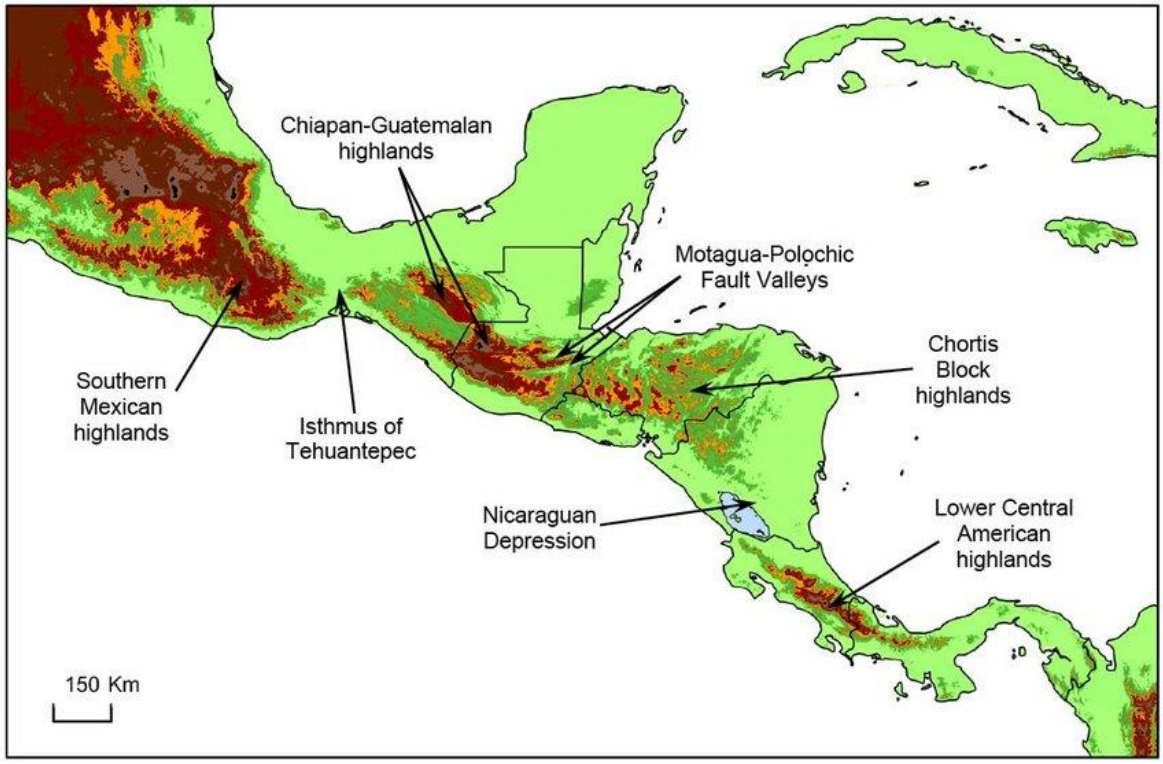

Figure 1. Map of the main biogeographical barriers and highlands between Southern Mexico and Central America (Castoe *et al.*, 2009).

Amphibian crisis

Amphibians are one of the most diverse groups in the world, with more than eight thousand described species that inhabit a variety of ecosystems. Unfortunately, at least 41% of the species are threatened, making them one of the most endangered vertebrate groups in the world (IUCN 2020). In 1996 the IUCN reported 18 critically endangered species, by 2019 this number had gone up to 575 species. For the endangered and vulnerable classifications, the numbers went from 31 to 944 and 75 to 638 species respectively. Despite those increases, 61% of the described amphibians in the world have either not been assessed by the IUCN Red List or are classified based on assessments that have not been updated in more than 10 years (Tapley *et al.* 2018). This is problematic for a group with such a high level of known threats.

Tropical regions have the highest levels of amphibian diversity, however in the past three decades the populations have been declining rapidly. In Mesoamerica they are the most endangered group with 38% of the species threatened, of which 23% are endemic to the region (IUCN 2020). Several populations have decline rapidly and some species have gone extinct e.g. *Craugastor milesi* from Honduras and *Bufo periglenes* from Costa Rica (Stuart *et al.* 2008). These declines have been associated with accelerated habitat destruction, pesticide pollution, climate change, pet trade and emergent diseases, like *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal) (Berger *et al.* 1998; Lips *et al.* 2005; Stuart *et al.* 2004; Mendelson III *et al.* 2006; Whitfield *et al.*, 2007; Wake & Vredenburg, 2008; Martel *et al.* 2013).

Amphibians are an important part of an ecosystem, they are the most abundant vertebrate by biomass (Semlitsch *et al.* 2014) and are the top predator of invertebrates (Hocking & Babbitt 2014; DuRant & Hopkins 2008). Research has demonstrated that the decline of amphibian species can affect the function of the ecosystems, and as a result the human well-being (Crump 2009). They are bioindicators of good habitat quality (Lee 2000) and contribute to control the numbers of crop pests and vector transmitting insects (DuRant & Hopkins 2008; Hocking & Babbit 2014; Valencia-Aguilar *et al.* 2013), which is important especially in tropical areas where diseases like malaria, zika and dengue affects a lot of communities.

Conservation genetics

Conservation genetics is the discipline that aims to apply genetics and molecular biology to biodiversity conservation (Frankham, 2010). Biodiversity can be studied at three levels, ecosystems, species and genes (Convention on Biological Diversity, 2001). Usually conservation efforts focus more on species and ecosystems, and diversity has been quantified by creating species inventories or identifying threatened species within an area (Coates *et al.*, 2018). While these can give us important information about the ecosystem and its function, it might not be able to detect abrupt changes due to habitat disturbance and fragmentation (Monteiro *et al.*, 2019). Genetic tools can give

us higher resolution data to study populations and to detect these changes in an ecosystem (Manel & Holderegger, 2013; Monteiro *et al.*, 2019).

Genetic diversity can have an influence on an ecosystem dynamics and its diversity (Frankham, 2010), thus it is important to focus conservation efforts on studying the genetic and evolutionary processes of species and populations. This type of approach can help to inform about the status of a population, and delimit populations, conservation units or species (Storfer, 2003; Coates *et al.* 2018). It is important to study not only the ecology of the populations but their genetic diversity as well, to be able to create conservation plans according to the needs of different, and potentially distinct, groups.

Learning about the evolutionary history and patterns that have shaped diversity is a way to efficiently help to understand and preserve the evolutionary processes. Conservation approaches should be improved by integrating information from molecular genetic analysis into ecological and phenotypic studies to be able to apply solutions to several species. The main goal should always be to preserve as much as we can, either to preserve the genetic diversity in endemic sites or the evolutionary processes that generate that diversity (Moritz *et al.*, 2000).

Molecular genetics can help scientists to resolve conservation issues like resolving taxonomic uncertainties (Hutter & Guayasamin, 2015; Moritz *et al.* 2018) and defining Conservation Units (CUs) for the optimal management of species (Coates *et al.*, 2018). To generate information about wild populations, like the presence of rare species (Rodgers *et al.*, 2017), their demographic history, associated microbial communities and diet (Beddek *et al.*, 2018; Dutton *et al.*, 2013; Francisco *et al.* 2018; Nolasco-Soto *et al.* 2017), as well as information about inbreeding and loss of genetic diversity (O'Brien, *et al.* 1985; O'Brien *et al.* 2017).

Captive populations and reintroduction programs can also benefit from these types of analysis, especially when breeding programs are being planned to identify the best individuals to breed and avoid inbreeding. Including genetic data when planning reintroductions or translocations is a necessary step to ensure a positive outcome

(Tzika, *et al.* 2009; Shan *et al.*, 2014; Jangtarwan *et al.*, 2019). Successful wildlife forensics programs have used genetics to trace back which species people trade in markets, or where the animal or parts of it was collected (Baker *et al.*, 2010; Harper *et al.*, 2018).

Microsatellites

Microsatellites or single sequence repeats (SSRs) are sequences of DNA consisting of motifs of one to six nucleotides repeated in tandem (Chistiakov *et al.* 2006; Selkoe & Toonen, 2006). They are codominant, highly informative and very variable markers that can be found in the nuclear genomes of most taxa (Selkoe & Toonen, 2006; Vieira *et al.* 2016), which makes them ideal for studying population genetics, diversity, parentage and to identify individuals.

Microsatellite markers are useful tools for conservation genetics, by having high mutation rates per locus per generation they can give information about recent events in a population, the genetic distinctiveness of the individuals and if there is gene flow among different populations or groups (Selkie & Toonen 2006; Jehle, 2010), which may be especially important in areas with high levels of fragmentation or for species that do not migrate long distances.

Cryptic species and Conservation Units (CUs)

Cryptic species are morphologically similar species that cannot be distinguished by their phenotype, but molecular genetics data indicates that they should be split into different evolutionary lineages (Rannala, 2015; Struck *et al.*, 2018). It is important to identify cryptic species to be able to increase the efficacy of conservation programs and preserve species (Struck *et al.*, 2018). Cryptic species are very common in herpetology (Crawford, *et al.* 2013; González-Porter *et al.*, 2013; Ye *et al.*, 2013; Suárez-Atilano *et al.*, 2014; Arteaga *et al.*, 2016). This is perhaps because, in the case of amphibians, several species communicate using non-visual signals (i.e. calls) that do not depend on the morphology of the individuals to diversify (Stuart *et al.* 2006; Bickford *et al.* 2007).

Studying cryptic species can be difficult and sometimes there is not enough data to split the complex into several species. Conservation units (CUs) are population units identified within species, they are useful to preserve populations and to guide the conservation efforts (Funk *et al.* 2012). By identifying CUs, conservation efforts can focus on preserving, or give priority to, the populations with the highest diversity or the ones that need more urgent actions to assure the survival of the species. Different CUs can be managed according to the needs of each one.

Conservation units can be divided into evolutionary significant units (ESUs) and management units (MUs) (Funk *et al.*, 2012). MUs are small units or populations that are demographically independent. MUs can be contained within ESUs and their preservation is important to assure the long term conservation of a species (Funk *et al.*, 2012). Evolutionary significant units (ESUs) are populations or a group of populations with a high genetic differentiation compared to others and should be managed separately to be able to guarantee their conservation (Ryder, 1986; Moritz, 1994; Funk *et al.*, 2012). By having different ESUs the capacity of a species or population to evolve is maximized, which is important when populations face sudden environmental changes (Funk *et al.*, 2012). Knowing the ESUs is also important for a conservation program to prioritize areas or populations, when resources are limited.

Habitat fragmentation is one of the main causes of amphibian decline around the world. Studies have determined that pond-breeding amphibians are very susceptible to fragmentation due to changes in land use and urbanization (Homola, *et al.* 2019). When a habitat is fragmented there will be little or no geneflow among the populations, resulting in low genetic diversity and inbreeding that could lead to the extinction of the species (Frankham, 2010). Hence the importance of gathering genetic data during the development of conservation plans, management of wild populations, identifying conservation units and threats assessment. Nevertheless, it is important to realize that in order to conserve amphibian populations it is not enough to gather genetic information if it is not integrated and used in conservations plans (Jehle, 2010). Knowing the conservation status of a species can help not only to conserve it as a group, but also the ecosystem as a system and therefore the ecosystem services it provides (Valencia-Aguilar *et al.*, 2013).

Aims of the PhD

This project had two central aims examined using both ex-situ and in-situ components. The first aim was to determine if there are genetic and phenotypic differences between the populations of *Agalychnis moreletii* to inform decisions for its conservation across its entire distribution. An associated aspect of this aim was to describe aspects of the species' ecology, skin microbiome and presence of diseases in Guatemalan populations.

The second aim was to characterize the skin-bacterial communities associated to *Plectrohyla matudai* and *Bolitoglossa* spp. from the forests of La Union, Zacapa in Guatemala. To record the changes that these communities undergo once the animals are moved from the wild into captivity, and to gather information about the husbandry needs of these genera.

Both aims had the objective of generating information for improving the conservation of amphibians in Mesoamerica and to promote the use of molecular tools to study biodiversity and ecology.

Overview of the chapters

In the second chapter I present the results of the design of microsatellite markers for *Agalychnis moreletii,* and the populations genetic analysis of this species. Populations from the Pacific coast have very low genetic diversity, mainly due to the destruction and habitat fragmentation. While populations of the Atlantic coast showed the highest diversity and connectivity. Data shows that there is a high population differentiation between populations of Veracruz, Atlantic and Pacific coasts. These should be considered three different evolutionary significant units for conservation purposes.

The third chapter describes the mitochondrial diversity in several populations of *Agalychnis moreletii* and how this can explain more about its past and long-term history. As in chapter 2, results show that there are at least 3 clusters or groupings in which the populations should be split for its management, Pacific, Atlantic and Veracruz.

The fourth chapter describes the characterization of the skin-bacterial communities of *Agalychnis moreletii* in Guatemala and how this could be influenced by the presence of *Batrachochytrium dendrobatidis.*

The fifth chapter describes the characterization of the skin-bacterial communities from wild amphibians of the genera *Plectrohyla* and *Bolitoglossa* from La Unión, Zacapa; and how they could be impacted when animals are taken from the wild into captivity.

The sixth chapter includes the general discussion for the PhD where I talk about the importance of integrating genetics and molecular biology tools with ecological data to be able to make conservation plans accordingly to each species' needs.

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Chapter 2 - Population genetics of the Black-eyed frog *Agalychnis moreletii* (Duméril 1853)

Introduction

Amphibians are one of the most diverse groups in the world, with more than eight thousand described species around the globe (IUCN 2020). Tropical regions, like Mesoamerica have the highest levels of amphibian diversity (IUCN 2020). Unfortunately, at least 41% of the species are threatened, making them one of the most endangered vertebrate groups in the world (IUCN 2019). These declines are due to several threats that include accelerated habitat destruction, change in land use, pesticide pollution, climate change and emergent diseases, like *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal) (Berger *et al.*, 1998; Longcore, *et al.*, 1999; Stuart *et al.*, 2004; Lips *et al.*, 2005; Whitfield *et al.*, 2007; Wake and Vredenburg, 2009; Crump 2010; Martel *et al.*, 2013).

The genus *Agalychnis* comprises 15 species of colourful tree frogs within the Phyllomedusidae family. They inhabit forests from Mexico, through Central America, Colombia, Ecuador, Venezuela, Peru and Brazil (Frost 2015). Due to the high phenotypic and morphological diversity in this genus there is still debate about the taxonomic status of the different species (Duellman 2001). The potential for cryptic species has long been recognised based on variation in morphology and skin colouration for some of the *Agalychnis* species (Robertson & Zamudio, 2009; Robertson & Vega, 2011, Solano-Flórez, 2012). Studying species of the *Agalychnis* genus in Central America (Solano-Flórez 2012) concluded that for conservation purposes *A. callidryas* and *A. lemur* should be treated as independent evolutionary units across its distribution range. For this study we focus on the black-eyed tree frog, *Agalychnis moreletii*, since it has a wide distribution in five countries and the genetic information about it is very limited.

Agalychnis moreletii inhabits pre-montane to montane moist forests of Mexico, Guatemala, Belize, El Salvador and Honduras (Figure 1) (IUCN 2017), its elevation range goes from 200 to 2130 mts. asl (Köhler 2011). It can be found breeding in natural ponds and wetlands as well as in man-made ponds in sites where the habitat has been severely degraded.

The species was included in the appendix II of the CITES index due to pet trade (CITES 2010). In 2016 it was taken from Critically Endangered to Least Concern by the IUCN Amphibian Specialist Group since they argue that the population decline predicted in 2004 could not be demonstrated with the resources and information available at the moment (IUCN 2017). Nonetheless they agreed that the population trend is decreasing, that there are several recognized threats for the species (e.g. loss of habitat, pollution, diseases) and that there is need for research and monitoring (IUCN 2017).

Figure 1. Map of the distribution of *A. moreletii*, shaded orange zones represent the areas where the species is distributed (AmphibiaWeb 2015).

Cryptic species are morphologically similar species that cannot be distinguished by their phenotype, but molecular genetic data indicates that they should be split into different evolutionary lineages (Rannala, 2015; Struck *et al.*, 2018). There are several

examples of cryptic speciation in herpetology (Crawford, *et al.* 2013; González-Porter *et al.*, 2013; Ye *et al.*, 2013; Suárez-Atilano *et al.*, 2014; Arteaga *et al.*, 2016).

It is critical that species complexes are identified and accounted for when creating a conservation strategies. For example, a species with a large distribution range could actually be a series of cryptic species where for each of those species the distribution range is actually restricted, and the effective population size is much smaller. It is only by linking genetic and ecological data that we can determine which areas and species need priority in conservation programs.

Evolutionary significant units (ESUs) are populations or a group of populations with a sufficiently high genetic differentiation from other groups that they should be managed separately for conservation (Ryder, 1986; Moritz, 1994; Funk *et al.*, 2012). By having different ESUs the capacity of a species or population to evolve is maximized, which is important when populations have to face sudden environmental changes (Funk *et al.*, 2012). Knowing the ESUs is especially important for a conservation program to prioritize areas or populations, when resources are limited.

Habitat fragmentation is one of the main causes of amphibian decline around the world. Studies have determined that pond-breeding amphibians are more susceptible to fragmentation due to changes in land use and urbanization (Homola, *et al.* 2019). When a habitat is fragmented there will be little or no geneflow among the populations, resulting in low genetic diversity and inbreeding that could lead to the extinction of the species (Frankham, 2010). When developing conservation plans it is important to include genetic data of the populations to be able to have a better picture of the population's genetic health.

The aim of this project was to determine if there are genetic differences among the populations of *Agalychnis moreletii* in Central America and México to identify conservation units and assess the status of the populations. As well as to establish the main factors causing these differences. The ecological and genetic information generated during this project will be important to improve conservation efforts of this and other species of *Agalychnis.*

Materials and Methods

Field surveys

Agalychnis moreletii surveys were done during the rainy season of 2016, 2017 and 2018 in several localities of Mexico, Guatemala, Belize, El Salvador and Honduras (Figure 2, Table 1). For identifying the sites where we could find *A. moreletii* we reviewed historical records and contacted scientists and natural reserve managers. We also explored potential new sites searching for tadpoles, eggs or any indication that *A. moreletii* was present.

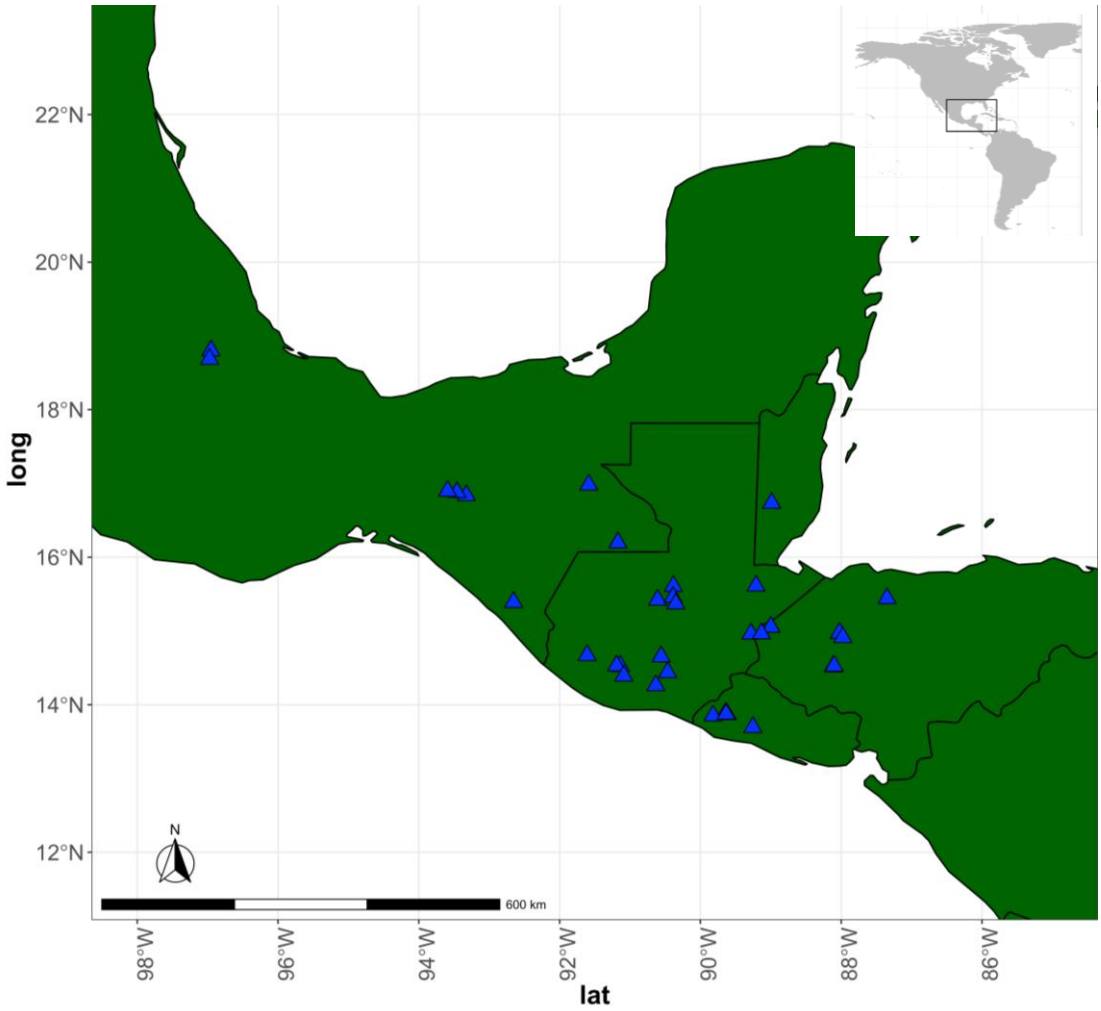


Figure 2. Sites were surveys for *A. moreletii* were done in Mexico, Belize, Guatemala, Honduras and El Salvador.

Table 1. Sampling sites were *Agalychnis moreletii* was found.

Country	Site	Site code	Longitude	Latitude
Belize	Las Cuevas Research Station, Chiquibul Forest Reserve, Cayo	LCRS	-88.98734	16.73293
El Salvador	Sector Los Andes Caserío Buenos Aires - DM, Santa Ana	DM	-89.64411	13.88108
	Sector Los Andes - Orchidiary, Santa Ana	LAO	-89.62007	13.86929
	Sector Los Andes -DA, Santa Ana	DA	-89.62007	13.86929
	Parque Bicentenario, San Salvador	BIC	-89.25462	13.69414
	Finca Los Andes, Ahuachapan	APA	-89.62007	13.86929
	Sector Los Andes, Plan del Hoyo, Santa Ana	PdH	-89.63486	13.87414
Guatemala	Finca La Gracia, Escuintla	GRA	-91.08885	14.39093
	Finca El Patrocinio, Quetzaltenango	PAT	-91.608855	14.6694
	Los Andes, Suchitepequez	AND	-91.19041	14.52848
	Los Tarrales, Suchitepequez	TAR	-91.13628	14.52183
	Las Nubes, Alta Verapaz	NUB	-90.38386	15.60798
	Finca El Naranjo, Guatemala	GUA	-90.55786	14.65176
	La Unión, Zacapa	LU	-89.28194	14.96134
	Sac-Wach Ja, Alta Verapaz	SWJ	-90.60755	15.4203
	Rubel Chaim, Alta Verapaz	RCH	-90.34885	15.37053
	Chaaxalap, Alta Verapaz	CHA	-90.38867	15.45817
Honduras	San Jerónimo - Aldea La Rodadora, Intibucá	SIS	-88.11088	14.5184
	Barrio El Paraíso, Intibucá	EPA	-88.10084	14.52492
Mexico	Tierra y Libertad, Chiapas	TyL	-93.32378	16.8373
	Laguna Bélgica, Chiapas	LBEL	-93.45689	16.87913
	Emilio Rabasa, Laguna Pomarosa, Chiapas	ERP	-93.59147	16.89745
	Los Cacaos, Chiapas	CAC	-92.6652	15.38927
	Xonomanca, Veracruz	XON	-96.97227	18.686
	Tequecholapa, Veracruz	TEQ	-96.95126	18.79967
	Ejido La Democracia, Chiapas	DEMO	-91.17156	16.19653
	Naha, Chiapas	NAHA	-91.58504	16.98026

Surveys were carried out during the night from 7pm to 11 pm using the visual encounter survey method (VES). All specimens were captured using new sterile gloves and placed in a plastic bag that was labelled with an identification number. Data about the microhabitat, time of capture, GPS location and activity of the individual were recorded.

Saliva samples were taken using a sterile rayon swab (MW113, Medical Wire Equipment & Co. Ltd) and stored in a 1.5mL vial tube filled with absolute ethanol. When necessary for voucher specimens under licensing conditions, 2 adult specimens per population were euthanized using a diluted solution of lidocaine and a sample of the liver was dissected and stored in absolute ethanol. When collecting tadpoles, the individuals were euthanized as described above. Collected individuals were fixed in 10% formalin and stored in the Biological Reference Collection or Museum of each country: Universidad del Valle de Guatemala Reference Collections, Colegio de la Frontera SUR (ECOSUR), Chiapas, Mexico, Universidad Nacional Autónoma de Honduras, Universidad Nacional de El Salvador.

Microsatellite development

Microsatellite markers for *A. moreletii* were developed using Illumina Next Generation Sequencing and bioinformatics tools. Eight samples of *A. moreletii* from different localities of Guatemala and Honduras (Supp information, Table 1) were used. DNA was extracted using the DNeasy blood and tissue extraction kit (Qiagen). An Illumina®-Nextera™ DNA library kit was used for the library preparation. Paired-end sequencing (2x250bp) was performed on an Illumina MiSeq at the University of Manchester Genomic Technologies Core Facility.

The raw sequence files were analysed for quality and filtered using the Pal_filter bioinformatics tool (Griffiths *et al.*, 2016) in the Galaxy Centaurus server at the University of Manchester Core Bioinformatics Facility. We used Trimmomatic v.0.32 (Bolger *et al.*, 2014) implemented in Pal_filter with the following settings, Sliding window – window size = 4bp, quality = 20, leading = 3, trailing = 3, minlen = 50.

After filtering and quality control, the eight files from each sample were analysed using Pal Finder (Castoe *et al.*, 2012), which searches for 'potentially amplifiable loci', PALs (SSR loci and flanking PCR primer sites) through the genome. It uses the software Primer 3 (Rozen & Skaletsky, 2000) to design primers for the loci found. The Python script MiMi (Fox *et al.*, 2019) was used to optimise the design of the microsatellite markers. The output was a database with 2,711 potential primer pairs to amplify microsatellite loci.

Twenty-four primer pairs were chosen for testing. For more cost-effective fluorescent-labelling of PCR products, a universal primer sequence 'tail' was added to the 5' end of each forward primer, the universal primer was labelled with a fluorophore and included as a third primer in the PCR reaction, following the methods of Culley *et al.* (2016) and Blacket *et al.* (2012). The tails used were tail C (CAGGACCAGGCTACCGTG) from Blacket *et al.* (2012), T7term (CTAGTTATTGCTCAGCGGT) and M13 Modified B (CACTGCTTAGAGCGATGC) (Culley *et al.*, 2016). After testing the twenty-four primers only twenty-two primer pairs showed clear bands on the agarose gels. These primers were used to design PCR multiplexes using the software Multiplex Manager 1.0 (Holleley & Geerts, 2009) (Table 2).

Sample processing

DNA was extracted from liver tissue and swabs using the DNeasy Blood and Tissue Kit (Qiagen). PCR mixes were made using Type-it® Microsatellite PCR Kit (Qiagen) to a final volume of 5ul, with 2.4ul of Master Mix, 1.2ul of water, 0.5ul of primer mix (2uM) and 1 ul of DNA. The PCR program conditions were 5 mins at 95°C, followed by 30 cycles of 30s at 95°C, 90s at 60°C, 30s at 72°C and a final extension of 30 min at 60°C. The PCR products were prepared for genotyping by mixing 9ul of HiDi Formamide (Thermo Fisher Scientific), 0.2ul of GeneScan LIZ 500 size standard (Thermo Fisher Scientific) and 0.8ul of the PCR products. The plates were sent to Manchester University Genomic Facilities or Sheffield University Core Genomic Facility, for capillary electrophoresis analysis using the DNA analyser 3730 sequencer (Thermo Fisher Scientific).

Table 2. Multiplex PCRs used to study the population genetics of *Agalychnis moreletii*

Multiplex	Primer name	Forward sequence	Reverse sequence	Repeat motif	Tail-dye	Range
M 1	AM47*	AGTTCTGTGTGGAGC CCAGG	GGACCCACCTAAC GTTTGCC	AAAT(16)	BC-6FAM	319-350
	AM9*	ACATCCTCTCCCCACA TTGC	GAACAATAGGGTA CACTTACCATTGC	ATC(15)	BC-6FAM	410-434
	AM32	TCTCAGTGTTTCATT AGTCAGACCG	AACTGTCCCTTTA TGTGGAAAGC	AAAC(16)	T7-HEX	347-375
	AM30*	CCATATGTGGGACCT CACCC	CAGGGGAGATTGT TATGCACC	AT(16)	T7-HEX	430-484
	AM36*	TACTCCCTGTGCACG TCTCG	AGGTCACCATGCC AGACAGG	TC(12)	M13-PET	368-390
M 2	AM42*	AATCCAACGTTAGGC TCCCC	CACTCCCCTCTGAG CTGTGC	AC(22)	B C-6FAM	323-351
	AM18*	ATGTTACCCCGCAGG TATGG	TTCGCAGTAATAG GTCTGGGC	ATC(12)	BC-6FAM	410-430
	AM11*	GTGACTGAAAAGGTG ACCTAAGC	TCTGCCTACTTAT ACCAACAAGTTCC	ATCT(28)	T7-HEX	294-354
	AM37*	AGGTCATGATTCCCA GCAGC	CAAAACCATGCCG AAAGAGG	ATGG(16)	T7-HEX	352-364
	AM25*	TGCTTTGGAATATGT TGGCG	AATGTCCTGGGTG CTTATTGC	ATT(24)	M13-PET	341-371
	AM8*	AGATTCCGGATGGAA CATGG	CACTGGTCATAAC ACAGGAATGC	AT(12)	M13-PET	417-443
M 3	AM6*	GTCCCTGCCTATTCCA CTGC	AGGAGAAGGCTCC ACTCAGC	AAAT(20)	BC-6FAM	367-405
	AM4	GCTGGTATCAGGAAG AGACGC	GTAAGCTCGCAGA CCAAACG	AT(12)	T7-HEX	367-393
	AM40*	CAGCAGGAGACCTCA TCCG	CAGACCTGGTGCT TAAGGGG	ATT(18)	M13-PET	321-342
	AM23*	CCCAATTGTCACTTT ACTCATACAGG	CAAGAGTCCGACT TCCCAGG	AC(12)	M13-PET	384-430
M 4	AM33	ACTGAAACTGGAGCA AGGGC	CCGTAAAGTTTCA GGCCCC	TCC(12)	BC-6FAM	343-391
	AM24	ATGTCCGTCTGAGCT CCTGG	ACACTTGCCTCTCA CCCAGC	ATT(12)	T7-HEX	334-382
	AM7*	CCGCCCTTAAAGTTG GTCC	GATTACGTCTACA ACGCCAACG	TCC(12)	BC-6FAM	325-364
M 5	AM31*	CAAAGCTACAGTGGA TGGTGAGG	TGGGTGGTGAGTA TGGGAGC	AT(18)	BC-6FAM	323-347
	AM20*	CGGAGACTCGATTAC ACACAGG	TAGGCAGTCAACC ATCTCGC	ATC(12)	BC-6FAM	361-421
	AM3*	ATCGAGTGAGAGGAG CCAGC	TGTTGTTGCCTTG TCACTGC	AT(14)	T7-HEX	390-408
	AM16*	TTTGCAGAGAAGCAC CATCC	CCTTGGACTTATCC CAAACTGC	ATT(12)	M13-PET/ROX	361-418

BC-6FAM: Blacket C - 6-FAM;
T7-HEX:T7term-HEX;
M13-PET: M13-MODB (CACTGCTTAGAGCGATGC) - PET
***Microsatellite used for genetic analysis, For DAPC and Structure all the loci were used.**

Data Analysis

The fragments were scored using the RStudio (v 3.5.2) package Fragman 1.0.9 (Covarrubias-Pazaran *et al.*, 2016) and binned using the package MstatAllele 1.05 (Alberto, 2009). The presence of null alleles was estimated using FreeNA (Chapuis and Estoup, 2007), which utilizes the EM algorithm developed by Dempster, *et al.* (1977). Tests for deviation from Hardy Weinberg equilibrium (HWE) and linkage disequilibrium (LD) among loci were calculated in GENEPOP v. 4.6 (Rousset, 2008) with the following parameters: 10,000 dememorization number, 1,000 batches and 10,000 iterations per batch.

Genetic diversity was calculated for each locus and population. The mean number of alleles, effective number of alleles, expected and observed heterozygosity and inbreeding coefficient was calculated using GenoDive 3,01 (Meirmans & Van Tienderen 2004). ADZE 1.0 (Szpiech *et al.* 2008) was utilized to estimate the allelic richness and the private allelic richness rarefied to 10 individuals per population (smallest sample size), to correct for sample bias. We used INEST 2.2 to test for evidence of bottlenecking using a two-phased mutation model, using the default parameters. The software runs two tests to determine this, heterozygosity excesses taking into consideration allelic richness and the mean ratio of allelic richness to allelic size (M-Ratio deficiencies). Statistical significance was tested using the Wilcoxon signed-rank test implemented in the software, based on 1,000,000 permutations.

INEST 2.2 (Chybicki 2017) was used to estimate corrected F_{IS} since some loci presented null alleles. The software tests simultaneously for inbreeding and null alleles as both factors can cause an excess of homozygotes within a population (Chybicki 2017). To test for statistical significance, we compared the full model "nfb" (null allele, inbreeding and genotyping failure) with the "nb" model (null allele and genotyping failure). The models were run using the Bayesian Interacting Multiple Model using 500,000 MCMC and 50,000 burnin cycles. The Deviance Information Criterion (DIC) was used to for model comparison.

Pairwise genetic distances (F_{ST}) were calculated in Arlequin v 3.5.2.2 (Excoffier *et al.*, 2005), and in FreeNA with and without ENA correction for null alleles. Analysis of molecular variance (AMOVA) was performed in GenoDive (Meirmans & Van Tienderen 2004) using the infinite allele model with 10,000 permutations. We ran two analyses the first one using the populations as the highest hierarchical level and a second one grouping the populations into different clusters. The R package LEA (Frichot & François 2015) was used to determine the population structure. An admixture analysis (Pritchard *et al.*, 2000) was performed to determine the number of ancestral populations (k) that best explained the data (Frichot *et al.*, 2014)

A discriminant analysis of principal components (DAPC) was done in ADEGENET (Jombart 2008). This is a multivariate statistical method that can identify clusters of genetically similar individuals and detect spatial genetic patterns (Jombart *et al.*, 2010). Since it does not includes assumptions about HWE deviations or linkage disequilibrium, all the 22 loci were used to perform the analysis. Several DAPC analyses were run; the first one including all 19 populations of the data set (19 clusters), separate analyses using only Pacific coast populations (PAC), Atlantic coast populations (ATL) and Veracruz populations (VER) were also performed.

To test if the potential differences we could find between the populations were due to geographic distance or barriers, we performed an Isolation by Distance (IBD) test in the R package ade4 (Dray & Dufour 2007). To achieve this, we performed a Mantel test correlating a geographical distance matrix against the genetic differentiation (F_{ST}) matrix obtained in FreeNA with the ENA correction and the F_{ST} matrix calculated in Arlequin. To visualize the data, we use the package MASS (Venables & Ripley 2002).

Results

Only populations with 10 or more individuals were used for the population genetics analyses. In total we had 451 samples from 19 sites that were genotyped for 22 loci (Table 2, Figure 2). Some loci showed moderate evidence of null alleles for some populations (Supp information, Table 2). Four loci showed LD, and pairs that remained significant after Bonferroni corrections for multiple comparisons were removed from downstream analyses leaving 18 loci in total (Table 2).

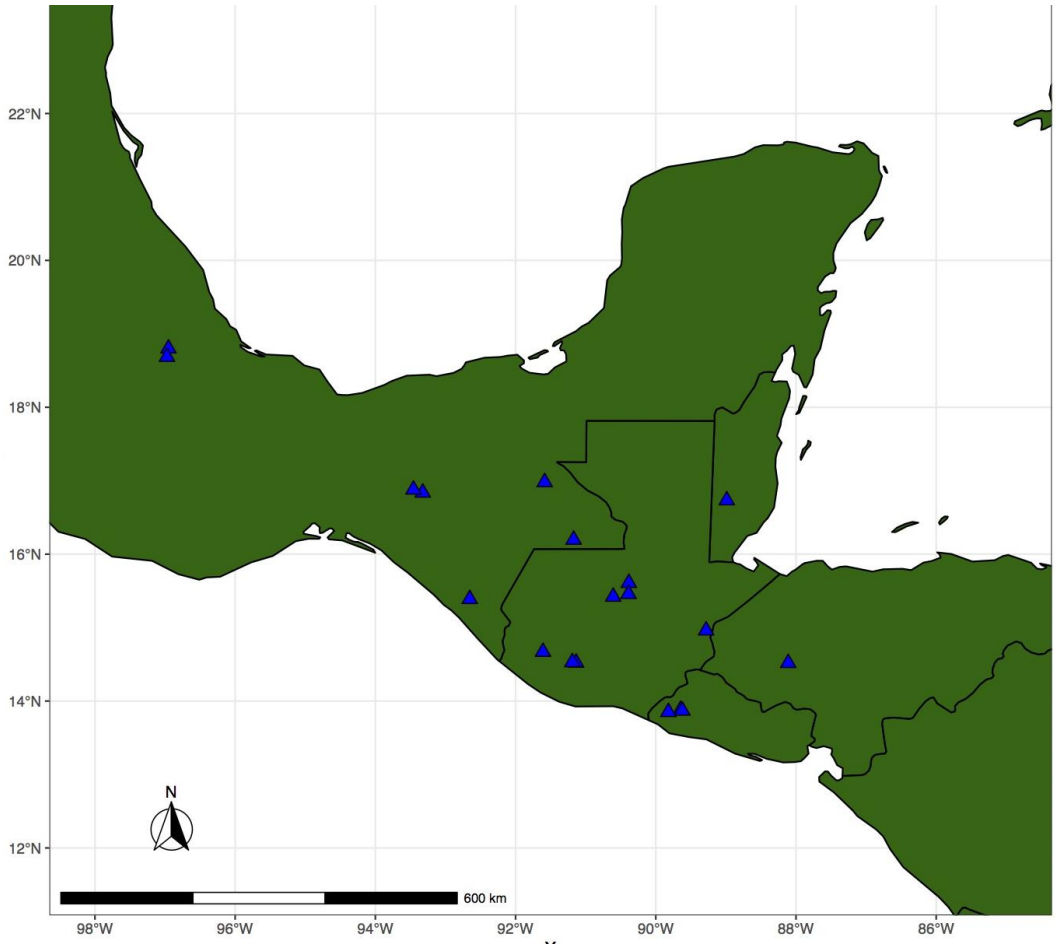


Figure 3. Map of the populations used for the genetics analyses.

Table 3. Sites used to do the population genetics analysis, abbreviations of site names, sample size and coordinates.

Country	Site	Site code	Sample size	Longitude	Latitude
Belize	Las Cuevas Research Station, Chiquibul Forest Reserve, Cayo	LCRS	52 ind	-88.98734	16.73293
El Salvador	Sector Los Andes Caserío Buenos Aires – DM, Santa Ana	DM	35 ind	-89.64411	13.88108
	Sector Los Andes – Orchidiary,	LAO	10 ind	-89.62007	13.86929
	Finca Los Andes, Ahuachapan	APA	13 ind	-89.62007	13.86929
Guatemala	Finca El Patrocinio, Quetzaltenango	PAT	18 ind	-91.608855	14.6694
	Los Andes, Suchitepequez	AND	20 ind	-91.19041	14.52848
	Los Tarrales, Suchitepequez	TAR	17 ind	-91.13628	14.52183
	Las Nubes, Alta Verapaz	NUB	18 ind	-90.38386	15.60798
	La Unión, Zacapa	LU	29 ind	-89.28194	14.96134
	Sac-Wach Ja, Alta Verapaz	SWJ	17 ind	-90.60755	15.4203
	Chaaxalap, Alta Verapaz	CHA	13 ind	-90.38867	15.45817
Honduras	San Jerónimo – Aldea La Rodadora, Intibucá	SIS	63 ind	-88.11088	14.5184
Mexico	Tierra y Libertad, Chiapas	TyL	12 ind	-93.32378	16.8373
	Laguna Bélgica, Chiapas	LBEL	29 ind	-93.45689	16.87913
	Los Cacaos, Chiapas	CAC	27 ind	-92.6652	15.38927
	Xonomanca, Veracruz	XON	16 ind	-96.97227	18.686
	Tequecholapa, Veracruz	TEQ	26 ind	-96.95126	18.79967
	Ejido La Democracia, Chiapas	DEMO	13 ind	-91.17156	16.19653
	Naha, Chiapas	NAHA	23 ind	-91.58504	16.98026

The average number of alleles for each population varied from 1.78 (LAO) to 4.22 (NAHA) with a mean of 2.94; the number of effective alleles ranged from 1.20 (DM) to 2.60 (NUB) with a mean of 1.85. The mean rarefied allelic richness ranged from 1.63 (std 0.167) in DM to 3.59 (std 0.213) in SWJ. The mean rarefied allelic richness of private alleles varied from 0.0038 in DEMO to 0.29 in NAHA. The mean expected and observed heterozygosity were 0.371 and 0.267, respectively (Table 4). The number of alleles per locus ranged from 2 (AM20) TO 14 (AM23 and AM11) with a mean of 9.11

(Supp info, Table 3). Overall the mean of the observed heterozygosity was significantly lower than the mean of the expected heterozygosity ($t = 7.62$, $df = 17$, $p > 0.0001$).

Table 4. Genetic diversity of alleles per population

Pop	Na	Ne	Ar	Pa-Ar	Ho	He	F
PAT	2.39	1.43	2.39	0.11	0.178	0.240	0.257
TAR	2.89	1.76	2.79	0.06	0.277	0.349	0.205
AND	3.28	1.73	2.70	0.07	0.255	0.355	0.282
NUB	4.06	2.60	3.58	0.09	0.335	0.571	0.414
SWJ	3.44	2.49	3.59	0.15	0.332	0.560	0.408
LU	3.28	1.78	3.04	0.20	0.204	0.409	0.502
CHA	2.72	1.85	3.17	0.05	0.317	0.427	0.256
DM	1.89	1.20	1.63	0.02	0.114	0.124	0.081
LAO	1.78	1.34	1.87	0.05	0.157	0.183	0.140
APA	1.94	1.40	2.53	0.04	0.151	0.224	0.326
SIS	3.33	1.81	2.70	0.06	0.263	0.367	0.283
LCRS	4.06	2.43	3.18	0.08	0.342	0.495	0.309
TyL	2.72	1.93	2.68	0.11	0.329	0.444	0.260
LBEL	3.83	2.02	3.9	0.24	0.280	0.441	0.364
CAC	2.17	1.43	1.89	0.11	0.181	0.213	0.152
DEMO	2.83	1.89	2.46	0.003	0.324	0.452	0.284
NAHA	4.22	2.48	3.34	0.29	0.420	0.527	0.203
TEQ	2.41	1.82	2.20	0.08	0.325	0.339	0.043
XON	2.59	1.72	2.21	0.08	0.322	0.333	0.032

N: number of alleles; Ne: effective number of alleles; Ar: rarefied allelic richness; Pa-Ar: rarefied private allelic richness; Ho: observed heterozygosity; He: expected heterozygosity; F: inbreeding coefficient. Abbreviations of site names can be found on Table 3.

DIC analysis in INEST showed that the populations AND, TyL, TEQ, XONO followed a "nb" MODEL and the rest of the populations followed an "nfb" model as the best fit for these sites (Table 5), suggesting that inbreeding is an important factor in these populations. The null allele corrected inbreeding coefficients ranged from 0.010 in TEQ to 0.326 in LU (Table 5). The posterior 95% probability intervals include zero in several populations (PAT, DM, LAO, LCRS), which means that F_{IS} cannot be significantly above zero and there is no sufficient evidence to say that there is inbreeding in those populations. The populations PAT ($p = 0.029$), NUB ($p = 0.008$), LU ($p = 0.008$), LCRS ($p = 0.0202$) and CAC ($p = 0.0202$) showed significant results for the bottleneck analysis following the M-Ratio method. This is evidence that an abrupt event has

caused a sudden loss of the allele diversity. In most populations it is believed that this was done to severe habitat loss causing population decline.

Since there was moderate evidence of null alleles ENA corrected Fst values are reported (Table 7). The F_{ST} values ranged from 0.024 (NUB-SWJ) to 0.687 (CHA-DM). Overall the F_{ST} values calculated in Arlequin (Supp info, Table 4) and in FreeNA were very high indicating high genetic differentiation and population structure along *A. moreletii* distribution range. For each locus the corrected F_{ST} values were also high (Table 6). When performing the AMOVA only with the populations as the highest hierarchical level the results showed that most of the variation occurs within individuals (43%) and among populations (40%). When grouping the populations into three different clusters we found that the highest percentage of variation is due to differences among the clusters and then variation within populations (Table 8).

Table 5. Corrected inbreeding coefficient and posterior 95% probability intervals for each population.

Pop	Avg(F_{IS})	95% HDPI
PAT	0.084	0 – 0.219
TAR	0.099	0.031 – 0.214
AND	0.085	0 – 0.236
NUB	0.112	0.043 – 0.212
SWJ	0.233	0.167 – 0.301
LU	0.326	0.254 – 0.401
CHA	0.160	0.138 – 0.196
DM	0.060	0 – 0.0136
LAO	0.082	0 – 0.232
APA	0.179	0.056 – 0.322
SIS	0.096	0.046 – 0.147
LCRS	0.056	0 – 0.114
TyL	0.065	0 – 0.179
LBEL	0.131	0.059 – 0.215
CAC	0.077	0.017 – 0.143
DEMO	----	----
NAHA	0.096	0.048 – 0.167
TEQ	0.010	0 – 0.034
XONO	0.033	0 – 0.096

Avg(F_{IS}): corrected inbreeding coefficient. 95% HDPI: posterior 95% probability intervals. Populations highlighted in yellow show the sites were the NB model was better fitted.

Table 6. Average number of null alleles per locus and global F_{ST} values with and without the ENA correction. Null allele frequency can be classify in three classes negligible (r < 0.05), moderate (0.05 ≤ r < 0.20), or large (r ≥ 0.20) (Chapuis and Estoup 2007).

Locus	Average null allele	Global F_{ST}	Global F_{ST} ENA correction
AM47	0.107	0.559	0.550
AM9	0.073	0.316	0.299
AM30	0.140	0.410	0.381
AM36	0.040	0.258	0.254
AM42	0.063	0.208	0.195
AM18	0.012	0.194	0.192
AM11	0.053	0.341	0.346
AM37	0.043	0.078	0.115
AM25	0.148	0.433	0.372
AM8	0.084	0.474	0.460
AM6	0.100	0.546	0.519
AM40	0.135	0.249	0.242
AM23	0.179	0.338	0.314
AM7	0.134	0.185	0.169
AM31	0.043	0.637	0.627
AM3	0.089	0.400	0.377
AM16	0.053	0.376	0.369
AM20	0.044	0.826	0.825

Table 7. F_{ST} values using the ENA correction in FreeNA. In bold are non-significant values.

	PAT	TAR	AND	NUB	SWJ	LU	CHA	DM	LAO	APA	SIS	LCRS	TyL	LBEL	CAC	DEMO	NAHA	TEQ	XON
PAT	-																		
TAR	0.090	-																	
AND	0.104	**0.016**	-																
NUB	0.384	0.330	0.322	-															
SWJ	0.422	0.354	0.346	0.024	-														
LU	0.504	0.450	0.442	**0.164**	0.152	-													
CHA	0.499	0.424	0.418	0.073	0.107	0.260	-												
DM	0.415	0.321	0.286	0.580	0.616	0.652	0.687	-											
LAO	0.292	0.195	0.179	0.433	0.462	0.546	0.559	**0.044**	-										
APA	0.238	0.132	0.115	0.407	0.439	0.521	0.527	0.071	**0.037**	-									
SIS	0.515	0.484	0.476	0.174	0.223	0.185	0.268	0.636	0.562	0.546	-								
LCRS	0.415	0.380	0.373	0.046	0.092	0.155	0.117	0.549	0.459	0.436	0.193	-							
TyL	0.493	0.405	0.402	0.179	0.230	0.280	0.246	0.664	0.535	0.500	0.310	0.233	-						
LBEL	0.462	0.400	0.399	0.159	0.215	0.244	0.210	0.622	0.511	0.487	0.258	0.199	0.060	-					
CAC	0.287	0.260	0.238	0.445	0.482	0.542	0.538	0.581	0.502	0.446	0.537	0.458	0.553	0.506	-				
DEMO	0.447	0.399	0.386	0.087	0.088	0.234	0.113	0.645	0.530	0.500	0.264	0.121	0.269	0.207	0.500	-			
NAHA	0.382	0.322	0.306	0.090	0.083	0.180	0.163	0.545	0.419	0.389	0.240	0.145	0.156	0.168	0.429	0.099	-		
TEQ	0.400	0.381	0.370	0.340	0.381	0.461	0.414	0.575	0.479	0.449	0.484	0.390	0.402	0.397	0.441	0.391	0.286	-	
XON	0.420	0.389	0.377	0.331	0.363	0.463	0.427	0.618	0.507	0.479	0.503	0.394	0.402	0.397	0.478	0.376	0.268	0.117	-

0 to 0.05 little genetic differentiation
0.05 to 0.15 moderate genetic differentiation
0.15 to 0.25 great genetic differentiation
>0.25 very great genetic differentiation

(Wright 1978; Hartl & Clark 1997).

Table 8. Analysis of molecular variance (AMOVA) for the different populations and clusters

Grouping	Source of Variation	d.f.	%var	Variance Comp	F-stat	F-value	Std. Dev.
Pops	Within Individual	---	43%	2.39	F_{IT}	0.571	0.055
	Among Individual (Pop)	---	17%	0.93	F_{IS}	0.280	0.069
	Among Populations	---	40%	2.25	F_{ST}	0.038	0.038
Clusters	Within Population	432	38%	3.73	Rho_{ST}	0.622	---
	Among Population	16	21%	2.09	Rho_{SC}	0.360	---
	Among Clusters	2	41%	4.05	Rho_{CT}	0.410	---

Both LEA and DAPC results showed three separated clusters (k) (Figures 4, 5). Atlantic coast populations (ATL), Pacific populations coast (PAC) and Veracruz populations (VER). A DAPC using only the Pacific coast and the Atlantic coast populations was run separately to see if there was further subdivision between the populations. For PAC populations we found that they subdivided into 3 different sub-clusters (Figure 6a), one only containing CAC from Mexico, the other one PAT, TAR and AND from Guatemala and the last one APA, LAO and DM from El Salvador. ATL populations subdivided into 3 sub-clusters comprising populations from 4 different countries (Figure 6b). One sub-cluster comprised populations of LU (Guatemala) and SIS (Honduras), the other one included the Mexican populations of LBEL and TyL. All the other populations grouped in the third sub-cluster.

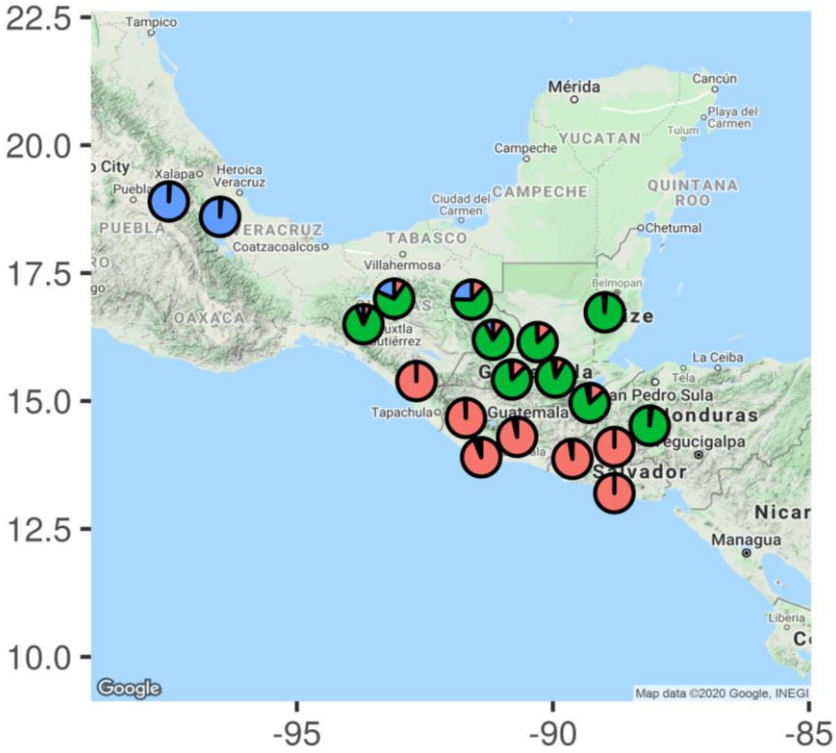


Figure 4. LEA analysis showing k=3. Red: PAC populations, Green: ATL populations, and Blue: VER Populations.

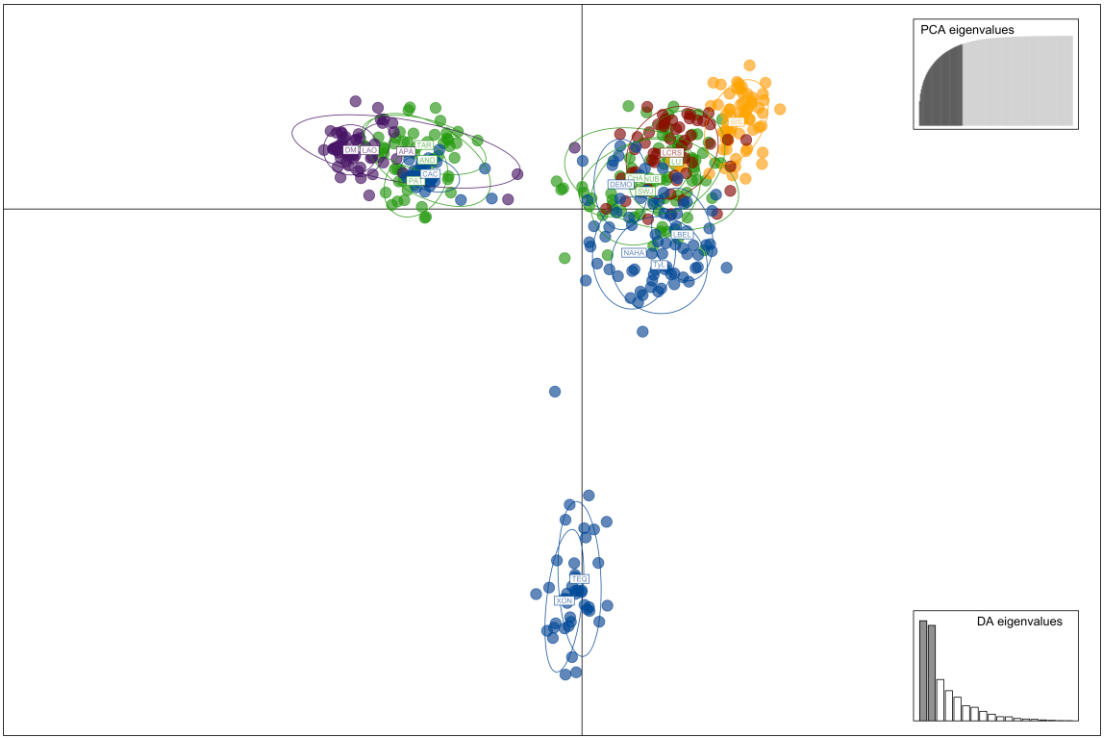


Figure 5. Discriminant analysis of principle components (DAPC) performed on all sites. The colours indicate the country where each population is located in: Green – Guatemala; Red – Belize; Purple – El Salvador; Yellow – Honduras; Blue – Mexico.

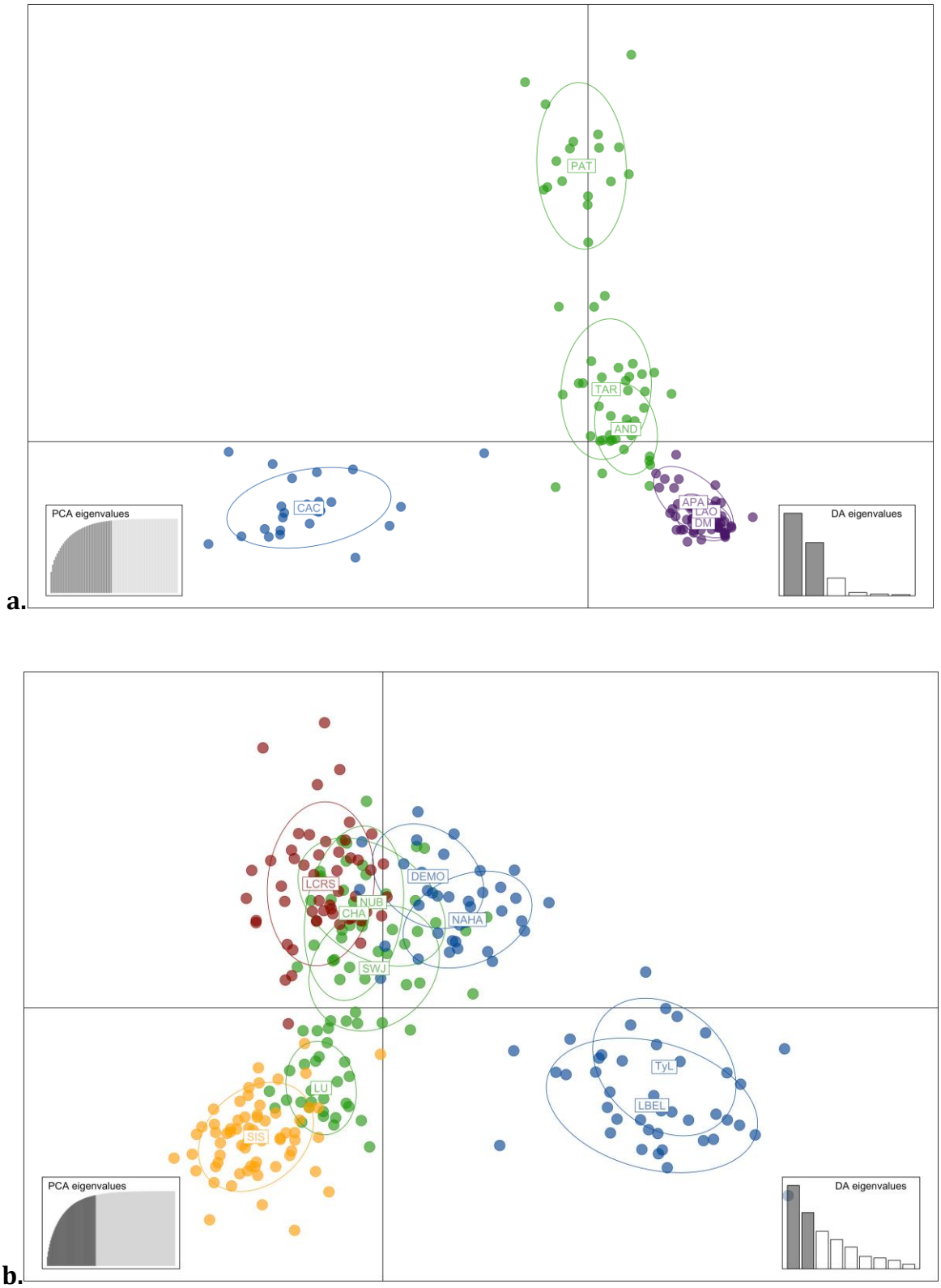
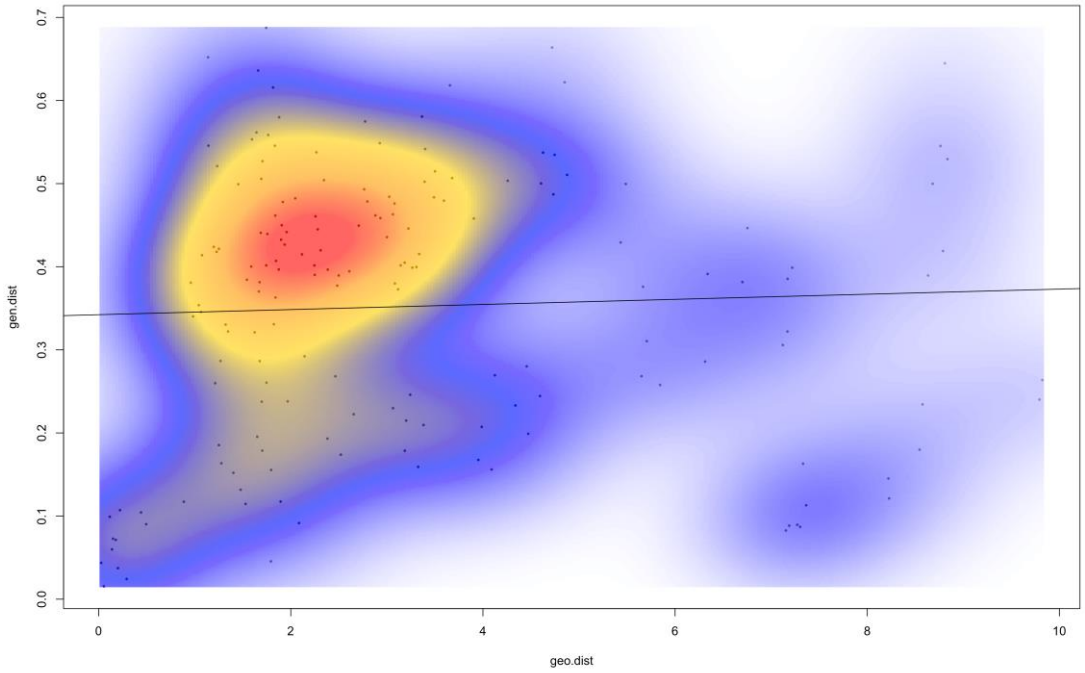
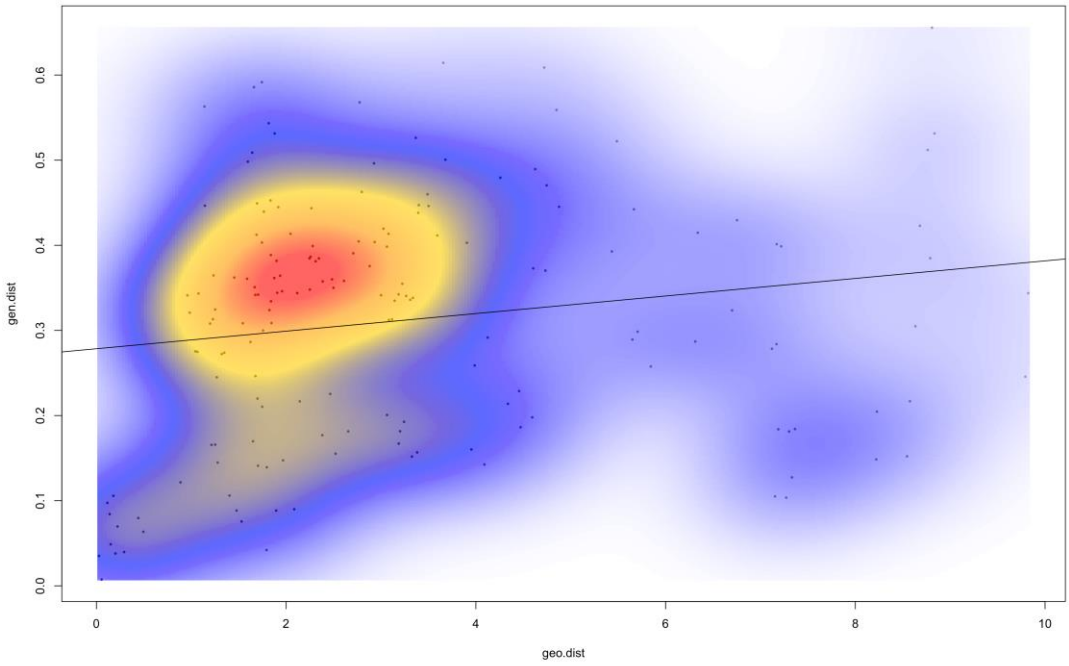


Figure 6. Discriminant analysis of principle components (DAPC) performed with the populations of the **a.** Pacific coast and **b.** Atlantic coast. The colours indicate the country where each population is located in: Green – Guatemala; Red – Belize; Purple – El Salvador; Yellow – Honduras; Blue – Mexico.

A Mantel test was performed to determine if the genetic differences observed were due to isolation by distance (IBD). We ran two tests one using the ENA corrected F_{ST} values from FreeNA and the F_{ST} values obtained in Arlequin. We could not find any significant relationship using any of the 2 genetic F_{ST} matrices (ENA corrected: r = 0.044; p-value= 0.31; ARLEQUIN: r = 0.17; p-value= 0.09) between geographic and genetic distances. When results were plotted, we did not find a consistent pattern in the cloud of points, meaning that data does not follow an IBD model (Figure 7).



a.



b.

Figure 7. Plots showing the Mantel test results **a.** Using the ENA corrected F_{ST} values; **b.** Using the F_{ST} values obtained in Arlequin. There was no significant relationship between distance and genetic differentiation. The different colours show discontinuous clouds of points, showing no IBD pattern.

Discussion

Agalychnis moreletii surveys were done in 27 sites in Mexico, Guatemala, Belize, Honduras and El Salvador. In general terms we found that the populations are very spatially isolated, likely due to habitat destruction or fragmentation. *Agalychnis moreletii* is a pond breeder and the availability of natural ponds was almost non-existent in most places. In a lot of locations, the populations are resilient and have adapted to inhabit artificial ponds. However, in some locations that historically supported significant *A. moreletii* local populations were greatly reduced and we could not find more than 10 individuals.

In terms of genetic diversity, Atlantic populations showed the highest number of alleles per population, number of effective alleles and allelic richness. Most of these populations inhabit preserved forests within governmental protected areas or private land with some degree of protection. While in sampling sites in PAC and VER most of the populations were breeding in places that had been highly degraded by human activities, showing lower levels of genetic diversity. In these places the habitat is very fragmented as a result of agriculture, urbanization and road construction and this is the likely cause of reduced genetic variation in these areas.

The DIC analysis showed that for 14 sites the best model was the "nfb" indicating that inbreeding could be present at these populations. After obtaining the null allele corrected inbreeding coefficients results showed only positive F_{IS} values from 0.10 to 0.33, being the higher values evidence of an excess of homozygosity which, could lead to inbreeding. The populations SWJ and LU were the ones with the highest F_{IS} values. Both of these populations are very isolated from other known populations. In the case of SWJ frogs breed in a large natural pond, but the forest is fragmented, and potential breeding ponds are scarce. LU site is a new record for this species, unfortunately it is very isolated within a cattle farm, the only breeding site is an old cow trough within a minuscule forest patch. *A. moreletii* is a pond breeding species and in localities within fragmented forests or where habitat has been destroyed, individuals from one sub-

population will not have much opportunity to breed with individuals from another sub-population.

Five populations showed significant results for bottleneck signal (PAT, NUB, LU, CAC and LCRS). In the case of the first four populations there has been a lot of habitat destruction that could have caused a drastic reduction in the populations size and consequently inbreeding. PAT is located in the south of Guatemala where the forest has been destroyed due to sugar cane crops. In this site the species lives in small wells surrounded by coffee plantations, the site resides within a private protected area and thanks to a birdwatching initiative some remnants of these forests are protected. NUB is private land with some areas used for coffee and timber, there is some forest surrounding the land and various old cattle troughs where individuals can breed. At the same time the farms around it have destroyed and fragmented the forest for coffee and palm oil plantations. As discussed previously LU is an isolated population and the forest around the breeding site is very disturbed. CAC is situated in Chiapas, Mexico, this population is in great danger since there is very little forest surrounding the town. The only individuals found were under a road were the water gathers during the rainy season, with a few trees surrounding it.

Finally, LCRS is a protected forest in the Chiquibul area in Belize, of all the sites visited during this project LCRS is the location were *A. moreletii* is most protected and the forest connectivity is optimal for the species. However, the Chiquibul area is susceptible to hurricanes and has been impacted multiple times in the past. Hurricane Hattie in 1961(Friesner 1993) and Hurricane Iris in 2001 (Bridgewater 2012) have disturbed and changed the forests and may be the potential cause of the bottleneck signal. Nonetheless the forest is very well preserved and there are several ponds around the area where you can find a high diversity of amphibians in one night.

After performing the ENA correction for the F_{ST} values in FreeNA they remained high for most of the pairwise comparisons ranging from 0.024 (NUB-SWJ) to 0.687 (CHA-DM). The global corrected F_{ST} was 0.387. The suggested guidelines for the interpretation of F_{ST} are from 0 to 0.05 there is little genetic differentiation, 0.05 to 0.15 indicates moderate genetic differentiation; 0.15 to 0.25 indicates great genetic differentiation and >0.25 indicates very great genetic differentiation (Wright 1978;

Hartl & Clark 1997). The high F_{ST} values that we observed in our data are evidence of very high genetic differentiation and population structure among the populations from the Atlantic, Pacific and Veracruz populations. AMOVA results showed a high percentage of variance among clusters, which agrees with the high values of F_{ST}. STRUCTURE and DAPC analysis showed three separated clusters, Atlantic populations (ATL), Pacific populations (PAC) and Veracruz populations (VER). DAPC ATL and PAC clusters where run individually to determine if there was some kind of sub-division within the clusters. For PAC populations we found that these subdivided into 3 different sub-clusters containing populations of El Salvador, Guatemala and Mexico, respectively (Figure 5a). As discussed before *A. moreletii* habitat on the Pacific coast consists of very disturbed forest patches and ponds or wells within towns and neighbourhoods.

The ATL populations also grouped into 3 distinct sub-clusters comprising several populations from different countries (Figure 5b). The first includes LU in Guatemala and SIS in Honduras. *A. moreletii* has been reported in the forests adjacent to LU in the Honduran side however permission to enter this locality was not granted. Our data suggests that there could be genetic connectivity between forests, LU and SIS populations. The second sub-cluster includes populations from Alta Verapaz in Guatemala and the Selva Maya jungle in Mexico and Belize. The F_{ST} values among these populations of ATL indicated very little differentiation, showing that there could be genetic connectivity between them. The third sub-cluster includes LBEL and TyL two sites in Chiapas, Mexico that are somehow isolated by the Chiapan-Guatemalan Highlands. In TyL *A. moreletii* breeds in the community's water supply which is surrounded by trees and vegetation. LBEL is an ecological and educational reserve where A. *moreletii* and *A. callidryas* co-inhabit. The species are very well preserved in this area, and the lagoon and the forests are in a good state.

In addition to habitat destruction and forest fragmentation, there exist several biogeographical barriers in Mesoamerica which can explain the separation of this species. In Mexico the Tehuantepec Isthmus separates the VER populations from all of the other populations. ATL and PAC they are divided by the Motagua-Polochic Fault Zone and the Honduras Chortis Block highlands. Within the ATL populations (clusters 2 and 3) the Sierra Madre de Chiapas and Chiapan and Guatemalan highlands forms a

barrier between them. This diversification pattern has been recorded in other species (González-Porter *et al.*, 2013; Suárez-Atilano *et al.*, 2014; Nolasco-Soto *et al.*, 2017; Cano *et al.,* 2018). To test this a Mantel test was performed to determine if the genetic differences detected were due to isolation by distance, but we did not find any significant relationship.

According to Urbina-Cardona & Loyola (2008) only 18.99% of the potential range of this species is within a protected area. Many of the potential habitats indicated in their study have decrease due to urbanization especially in the Pacific coast of Guatemala, Mexico and El Salvador. The Ministry of Environment and Natural Resources (MARN) of El Salvador has found a new population near the capital city, where the construction of a new neighbourhood has been authorized, and not much has been done to protect the species habitat (pers. comm. MARN 2016). In Guatemala there is only 2% remaining of the forests of the Pacific coast leaving the species with almost no opportunities. In Veracruz populations were breeding in really small spaces with almost no natural habitat surrounding them. One of the ponds is situated next to a main road with no vegetation cover.

In 2016 the IUCN downgraded *A. moreletii* from Critically Endangered to Least Concern even though they recognized there was more need for research and monitoring. Given that our samples cover the entire range of this species, our results lead us to conclude that these groups should be managed as evolutionary significant units (ESUs) or even species. Dividing them as ATL populations, PAC populations and the VER populations being the later any population above the Tehuantepec Isthmus (Figure 2). By moving the species to least concern, we risk endangering the future of several populations which are already on the brink and without the attention afforded by endangered status are unlikely to receive the necessary support for them to survive. Even in sites with large populations, the majority showed evidence for inbreeding and homozygote excess. If there is any change in the environment the populations might not have sufficient genetic diversity to adapt successfully.

These further underlines the importance of genetic information in informing conservation plans allowing us to preserve unique populations and their genetic resources. It is crucial that conservation strategies are applied to each of these evolutionary units accordingly to the populations needs and threats. PAC and VER are

the most endangered ESUs and it is urgent to create conservation plans to improve the health of the populations. In the case of ATL populations is highly important to preserve sites like LCRS, NAHA, DEMO, SWJ and LBEL that showed high levels of diversity and are some of the last well conserved sites where *A. moreletii* is found.

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Supplementary information

Table 1. Samples of *Agalychnis moreletii* used for Illumina Sequencing and design of microsatellite markers.

Code	Country	Department	Site
JS2189	Guatemala	Guatemala	UVG
LS105	Guatemala	Suchitepéquez	Finca Los Andres
AM9G11	Guatemala	Guatemala	El Rio
JS1893	Honduras	Intibucá	Aldea La Rodadora
JS1894	Honduras	Intibucá	Aldea La Rodadora
JA1908	Honduras	Intibucá	Aldea La Rodadora
JS2073	Guatemala	Escuintla	Finca El Socorro
MEA	Guatemala	Izabal	El Estor

Table 2. Genetic diversity indices for each locus.

Locus	Num	Eff_num	Ho	Hs	Ht	H't	Gis
AM11	14	1.872	0.414	0.482	0.763	0.779	0.14
AM16	7	1.753	0.387	0.449	0.649	0.66	0.138
AM18	8	1.804	0.652	0.454	0.559	0.565	-0.436
AM20	2	1.1	0.034	0.1	0.49	0.512	0.655
AM23	14	1.795	0.152	0.467	0.676	0.689	0.674
AM25	13	1.893	0.273	0.494	0.788	0.805	0.447
AM3	13	2.093	0.419	0.548	0.858	0.876	0.236
AM30	13	1.712	0.228	0.435	0.727	0.743	0.474
AM31	6	1.276	0.176	0.225	0.712	0.739	0.219
AM36	9	1.345	0.222	0.265	0.349	0.354	0.164
AM37	4	1.076	0.048	0.074	0.079	0.079	0.344
AM40	8	1.517	0.166	0.356	0.464	0.47	0.534
AM42	10	2.369	0.524	0.599	0.772	0.782	0.126
AM47	6	1.327	0.1	0.259	0.453	0.464	0.613
AM6	8	1.603	0.253	0.392	0.762	0.783	0.354
AM7	10	1.602	0.285	0.392	0.473	0.477	0.272
AM8	12	1.597	0.262	0.39	0.703	0.721	0.329
AM9	7	1.393	0.207	0.293	0.389	0.394	0.292
Overall	9.111	1.618	0.267	0.371	0.593	0.605	0.28

Num= number of alleles, Eff_num= effective number of alleles, Ho= Observed heterozygosity, Hs=Heterozygosity within populations, Ht=Total heterozygosity, H't= corrected total heterozygosity, Gis= inbreeding coefficient.

Table 3. F_{ST} values obtained in Arlequin. Bold figures are non-significant.

	PAT	TAR	AND	NUB	SWJ	LU	CHA	DM	LAO	APA	SIS	LCRS	TyL	LBEL	CAC	DEMO	NAHA	TEQ	XON
PAT	0																		
TAR	0.063	0																	
AND	0.080	**0.007**	0																
NUB	0.308	0.272	0.274	0															
SWJ	0.325	0.276	0.275	**0.040**	0														
LU	0.385	0.344	0.346	0.145	0.106	0													
CHA	0.362	0.308	0.313	0.049	0.070	0.166	0												
DM	0.344	0.286	0.246	0.531	0.543	0.563	0.592	0											
LAO	0.217	0.170	0.141	0.382	0.389	0.447	0.439	**0.035**	0										
APA	0.147	0.088	0.076	0.309	0.300	0.365	0.342	0.106	**0.038**	0									
SIS	0.446	0.420	0.413	0.155	0.181	0.166	0.225	0.586	0.509	0.453	0								
LCRS	0.338	0.312	0.313	0.042	0.090	0.139	0.088	0.496	0.404	0.341	0.177	0							
TyL	0.405	0.342	0.335	0.167	0.201	0.229	0.193	0.609	0.470	0.373	0.298	0.214	0						
LBEL	0.376	0.336	0.340	0.152	0.182	0.198	0.157	0.559	0.445	0.370	0.258	0.186	0.084	0					
CAC	0.245	0.210	0.220	0.399	0.414	0.447	0.444	0.526	0.438	0.355	0.489	0.403	0.498	0.449	0				
DEMO	0.429	0.399	0.401	0.181	0.184	0.217	0.184	0.655	0.531	0.423	0.344	0.205	0.292	0.259	0.522	0			
NAHA	0.324	0.284	0.279	0.104	0.105	0.152	0.127	0.512	0.385	0.305	0.246	0.148	0.143	0.160	0.393	0.097	0		
TEQ	0.361	0.342	0.351	0.321	0.341	0.386	0.343	0.568	0.463	0.391	0.460	0.348	0.385	0.358	0.412	0.415	0.287	0	
XON	0.381	0.350	0.360	0.324	0.334	0.398	0.364	0.614	0.501	0.412	0.479	0.358	0.403	0.362	0.445	0.442	0.289	0.122	0

Chapter 3 - Phylogenetics of the Black-eyed frog *Agalychnis moreletii* (Duméril 1853)

Introduction

For several centuries biologists and naturalists have used morphology to categorize species. However using only morphological data does not always provide strong evidence to define a species, since speciation does not necessarily generate morphological changes in a population (Bickford *et al.* 2007; Hutter & Guayasamin, 2015). Cryptic species are two or more different groups that have been classified as one species due to morphologically similar characteristics (Bickford *et al.* 2007). Species like bats and some amphibians, that communicate utilizing non visual signals, are more likely to be cryptic species, because the diversification of the signals (acoustic, pheromones, etc.) do not necessarily involves a change in morphological features (Kingston *et al.* 2001; Bickford *et al.* 2007; Stuart *et al.* 2006).

In places with high levels of species diversity and endemism, like the tropics, it is crucial to have knowledge about its biodiversity and species delimitations. A cryptic species that originally had a large distribution range could actually be composed of several small population species with restrictive distribution ranges and without any protection status (Bickford *et al.* 2007; Funk *et al.* 2012; Moritz *et al.*, 2018; Juste *et al.* 2019). Different species need different conservation approaches and not having this information could have negative implications for the preservation of the species (González-Porter *et al.*, 2013; Hutter & Guayasamin, 2015; Garrick *et al.* 2018; Juste *et al.* 2019).

It is important to identify areas with high levels of diversity and endemism to be able to recognize new sites to preserve or areas that might need reassessment in order to protect the ecosystem and the species that inhabit it. Some species that are catalogued as endangered could actually be composed of several species with urgent

conservation needs (Schönrogge *et al.* 2002). Having a more accurate idea of the number of species in an area is also important in terms of cost-effectiveness, since scientists can focus the conservation efforts on populations that need it most or in a way that the needs of most populations are met.

Integrating molecular and genetic data with ecological and phenotypical information has been a more reliable way to determine if one species is actually a species complex that should be divided into several (Bickford *et al.* 2007; Fouquet *et al.* 2007). The use of molecular and genetics techniques to study biodiversity has helped to uncover species complexes in the wild (Funk *et al.* 2012; González-Porter *et al.* 2013; Arteaga *et al.* 2016) as well as in captive (Crawford *et al.* 2013) and in museum collections (Jin *et al.* 2020), some of these species might not exist in the wild anymore.

Using molecular biology several cryptic species of amphibians have been identified around the world (Vences & Glaw 2005; Funk *et al.* 2012; Hutter & Guayasamin 2012; Ye *et al.* 2013; Hutter & Guayasamin 2015; Arteaga *et al.* 2016; Cryer *et al.* 2019). Methods like DNA barcoding can be a valuable tool for recording amphibian diversity before populations decline or go extinct (Vences *et al.* 2005). If the actual number of amphibian species is underestimated the current crisis could be worse than previously believed (Fouquet *et al.*, 2007).

The amount of cryptic species is not known, especially in areas with high biodiversity that have not been explored widely (Funk *et al.* 2012). This is the case of Mesoamerica, a biodiversity hotspot comprising Central America, Mexico and the Caribbean. It is one of the most diverse areas in the world and in terms of amphibians the most diverse. Cryptic species complexes have been described in Central America (Wang *et al.*, 2008; Townsend *et al.*, 2011; Cryer *et al.*, 2019) and there are probably more yet to be described.

Black-eyed tree frog, *Agalychnis moreletii*

Agalychnis moreletii is a charismatic tree frog from the Phyllomedusidae family. It is distributed in pre-montane to montane moist forests from central Mexico all the way to the North western Honduras and Central El Salvador (IUCN 2017). Its elevation range goes from 200 to 2130 mts asl (Köhler 2011). It can be found in natural ponds and wetlands as well as in man-made ponds, especially in sites were the habitat has been severely degraded.

The populations of *A. moreletii* seem to be declining due to several threats like habitat destruction, pet trade and chytridiomycosis (Lips *et al.*, 2004; Lawson *et al.*, 2011; Kaiser & Pollinger, 2012; IUCN 2017). In 2010 the species was included in the appendix II of the CITES index (CITES 2010).

Agalychnis moreletii was classified as Critically Endangered by the IUCN Red List, but in 2016 it was downgraded to Least Concern even though the population trend is downwards and there is a need for more research about its population size, distribution and threats. Even when the species seems to be resistant to habitat degradation, the threats affecting them might increase in the future and cause the decline of its populations (IUCN 2017).

Despite being re-classified as Least Concern the population genetics and evolutionary history of *Agalychnis moreletii* has not been investigated. For this study samples of *A. moreletii* were collected along its distribution range. Using two mitochondrial genes (CytB and 16S) the phylogenetic and genetic diversity of the species was investigated. The aims of this chapter were to assess the genetic diversity within populations of *Agalychnis moreletii*, to determine the genetic structure and phylogeography among them and to define ESUs for the optimal conservation genetics management.

Materials and Methods

Field surveys

Agalychnis moreletii surveys were done during the rainy season of 2016, 2017 and 2018 in several localities of Mexico, Guatemala, Belize, El Salvador and Honduras (Figure 2, Table 1). Surveys were carried out during the night from 1900-2300 hrs using the visual encounter survey method (VES). All specimens were captured using new sterile gloves and were placed in a plastic bag that was labelled with an identification number. Data about the microhabitat, time of capture, GPS location and activity of the individual was recorded.

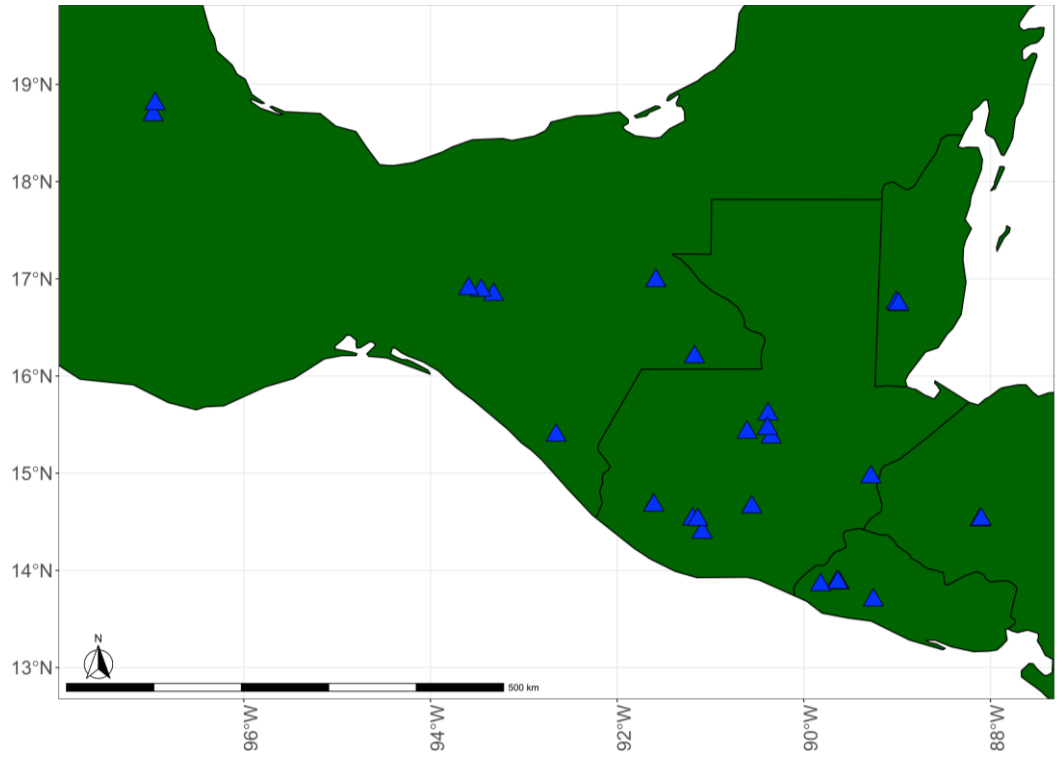


Figure 1. Sites were samples for *A. moreletii* were obtained.

Buccal swabs were taken using a sterile rayon swab (MW113, Medical Wire Equipment & Co. Ltd) and stored in a 1.5mL vial tube filled with absolute ethanol. When necessary 2 to 3 specimens per population were euthanized using a diluted solution of lidocaine and a sample of the liver was dissected and stored in absolute ethanol. The individual was fixed in formalin 10% and stored in the Biological Reference Collection or Museum from each country.

Table 1. Sampling sites for *Agalychnis moreletii.*

Country	Site	Site code	Longitude	Latitude
Belize	Las Cuevas Research Station, Chiquibul Forest Reserve, Cayo	LCRS	-88.98734	16.73293
El Salvador	Sector Los Andes Caserío Buenos Aires - DM, Santa Ana	DM	-89.64411	13.88108
	Sector Los Andes - Orchidiary, Santa Ana	LAO	-89.62007	13.86929
	Sector Los Andes -DA, Santa Ana	DA	-89.62007	13.86929
	Parque Bicentenario, San Salvador	BIC	-89.25462	13.69414
	Finca Los Andes, Ahuachapan	APA	-89.62007	13.86929
	Sector Los Andes, Plan del Hoyo, Santa Ana	PdH	-89.63486	13.87414
Guatemala	Finca La Gracia, Escuintla	GRA	-91.08885	14.39093
	Finca El Patrocinio, Quetzaltenango	PAT	-91.608855	14.6694
	Los Andes, Suchitepequez	AND	-91.19041	14.52848
	Los Tarrales, Suchitepequez	TAR	-91.13628	14.52183
	Las Nubes, Alta Verapaz	NUB	-90.38386	15.60798
	Finca El Naranjo, Guatemala	GUA	-90.55786	14.65176
	La Unión, Zacapa	LU	-89.28194	14.96134
	Sac-Wach Ja, Alta Verapaz	SWJ	-90.60755	15.4203
	Rubel Chaim, Alta Verapaz	RCH	-90.34885	15.37053
	Chaaxalap, Alta Verapaz	CHA	-90.38867	15.45817
Honduras	San Jerónimo - Aldea La Rodadora, Intibucá	SIS	-88.11088	14.5184
	Barrio El Paraíso, Intibucá	EP	-88.10084	14.52492
Mexico	Tierra y Libertad, Chiapas	TyL	-93.32378	16.8373
	Laguna Bélgica, Chiapas	LBEL	-93.45689	16.87913
	Biosphere Reserve Selva el Ocote, Laguna Pomarosa, Chiapas	POM	-93.59147	16.89745
	Los Cacaos, Chiapas	CAC	-92.6652	15.38927
	Xonomanca, Veracruz	XON	-96.97227	18.686
	Tequecholapa, Veracruz	TEQ	-96.95126	18.79967
	Ejido La Democracia, Chiapas	DEMO	-91.17156	16.19653
	Naha, Chiapas	NAHA	-91.58504	16.98026

DNA extraction and amplification

DNA extractions were done using the Blood and Tissue extraction kit (Qiagen). PCR reactions were done to amplify for the mitochondrial fragments 16S and Cytochrome B (CytB), using the primer pairs 16SA-L / 16SB-H (Palumbi 1991) and MVZ 15 / MVZ16 (Moritz *et al.* 1992). The PCR master mix was done to a final volume of 20 ul; using 9.6 ul MyTaq™ Red Mix (Bioline), 0.8ul of each primer (10uM), 6.8ul of PCR grade water and 2 ul of DNA.

The PCR program for 16S was 95°C for 3 minutes, followed by 25 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 20 seconds and a final extension of 72°C for 10 minutes. For CytB the program consisted of 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 46°C (CO1) or 48°C (CytB) for 30 seconds and 72°C for 30 seconds and a final extension of 72°C for 10 minutes.

DNA was visualized in 1% agarose gels and purified using the illustra™ ExoProStar™ (Merck) or ExoSAP-IT™ (ThermoFisher Scientific) kits following the manufacturer instructions. Cleaned PCR products were sent to Manchester University Genomic Facilities or Sheffield University Core Genomic Facility.

Data Analysis

Quality of the sequences was checked and aligned using Geneious Prime ® 2019.2.1 (Biomatters Ltd). 16S and CytB sequences were concatenated into one file, the final data set included the samples that amplified for both genes. The mutational model that best fit the data was identified using JModel Test 2.1.10 (Posada, 2008). Two models were run independently for 16S and CytB, a third mutational model was performed for the concatenated sequences file.

Phylogenetic relationships under the mutational model GTR+G+I were constructed using Bayesian Inference methods (BI) in MrBayes 3.2 (Ronquist *et al.* 2012) performing 100 million generations, sampling every 1000 generations, with a burning of 25%. Conversion was tested checking the plot (generated by sump

command) displaying the generation versus the log probability of the data (log likelihood values). The plot showed no clear trend or separation of the 2 runs. Conversion was also checked with the ESS (Effective Sample Size) value. All the values of ESS were >100 so we could conclude that models reached conversion.

Maximum likelihood (ML) methods tree was constructed using raxmlGUI 2.0 (Edler *et al.* 2019), running the model for 1000 bootstrap replicates to assess node support. Sequences from *A. annae* and *A. saltator* were used as outgroups. A third tree was constructed with the Neighbor-Joining (NJ) method performing 1000 bootstrap replicates in MEGA X (Kumar *et al.* 2018). Consensus trees were generated using FigTree v1.4.4 (Rambaut 2018). A node was considered well supported with a bootstrap value of 70% or greater for ML and NJ analysis. For Bayesian methods a posterior probability of 0.95 was a good indicative of robust support.

Relationship between haplotypes was inferred by constructing networks using the median joining method available in POPART (Leigh & Bryant 2015), with the default epsilon value set at zero. The two genes were treated separately as not all the individuals had sequences for both. DnaSP v6 (Rozas *et al.* 2017) was used to calculate diversity estimates for each lineage: the haplotype (Hd) and nucleotide diversity (π) and average number of differences among sequences (k) To determine if the sequences follow a neutral model of evolution the tests Tajima's D (Tajima 1989), Fu's Fs (Fu 1997) and Fu and Li's F (Fu and Li 1993) were performed on DnaSP v6, the coalescent simulator was used to test for statistical significance. The pairwise Φ_{ST} genetic distances were estimated on DnaSP v6.

Results

Genetic diversity

An alignment of 143 samples of 709 nucleotide positions was used to construct a median joining network for CytB (Figure 2a). CytB network resolved in 19 haplotypes with a total of 33 polymorphic sites. The average number of nucleotide differences was 2.202. For 16s we used 146 individuals of 536 nucleotide positions of length to construct the network. 16S analysis identified 12 different haplotypes, 12 polymorphic sites and an average number of nucleotide differences of 1.81 (Table 2). The haplotype and nucleotide diversity for CytB were 0.766±0.025 and 0.0105±0.0005, respectively. In the case of 16S the haplotype and nucleotide diversity were slightly lower, being 0.739±0.028 and 0.0057±0.0004, respectively (Table 2).

CytB analysis grouped the populations into four clades, Atlantic (ATL), Pacific (PAC), Veracruz (VER) and eastern Chiapas (CHI) populations. There was between 3 (ATL – CHI) to 15 (PAC-VER) mutational substitutions between the clades (Figure 2a). The most diversified cluster was ATL having 10 different haplotypes, followed by CHI with 5, PAC with 3 and VER with 1. All the haplotypes were found exclusively on each cluster. The populations of ATL and CHI had the highest values of nucleotide and haplotype diversity (Table 2).

The 16S lineage network grouped the populations into three clades, the first one contained 8 haplotypes exclusively found on ATL populations, showing the highest diversity. The second cluster included haplotypes found on PAC and CHI populations, 2 were exclusive of PAC. The third cluster contained only 1 haplotype exclusive to VER populations. There was between 1 to 2 mutational substitutions among the different clusters (Figure 2b).

Neutrality tests Tajima's D (CytB: D=-0.453; P > 0.10; 16SD=-0.576; P>0.10), Fu's Fs (CytB: Fs = -3.08; P > 0.10; 16S: Fs=-2.01 P>0.10) and Li's F test (CytB: F=-2.06; P >0.05; 16S: F=0.395; P>0.10) were not significant for either lineage, in indicating that the genetic variation between the populations is not under selection. For individual clades neutrality test were significantly negative for lineage CytB at ATL, which is consistent with population expansion (Table 2). Pairwise Φ_{ST} values indicated high genetic differentiation between most of the clusters for both lineages, only when comparing CHI-PAC the genetic difference was moderate (Table 3).

Table 2. Diversity estimates and neutrality test for CytB and 16S lineages

Lineage	Clade	N	S	h	Hd	π	*k*	T's D	FFs	F&L's F
CytB	***All***	143	33	19	0.767 ±0.025	0.0105±0.0005	5.202	-0.453	-0.308	-2.06
	ATL	59	13	10	0.510±0.072	0.0021±0.0004	1.030	-1.914*	-5.52*	-2.92*
	PAC	60	3	3	0.186±0.064	0.0045±0.0001	0.222	-1.30	-1.10	-1.92
	VER	12	0	1	0.00	0.00	0.00	NA	NA	Na
	CHI	12	5	5	0.742±0.096	0.0037±0.0008	1.288	-0.819	-1.20	-1.09
16S	***All***	146	12	12	0. 739±0.028	0.0057±0.0004	1.81	-0.576	-2.01	0.395
	ATL	64	9	9	0.66±0.059	0.0039±0.0006	1.32	-1.094	-2.43	-0.862
	PAC	58	4	4	0.304±0.073	0.0007±0.0002	0.42	-1.017	-1.70	-0.729
	VER	12	0	1	0	0	0	NA	NA	
	CHI	12	0	1	0	0	0	NA	NA	

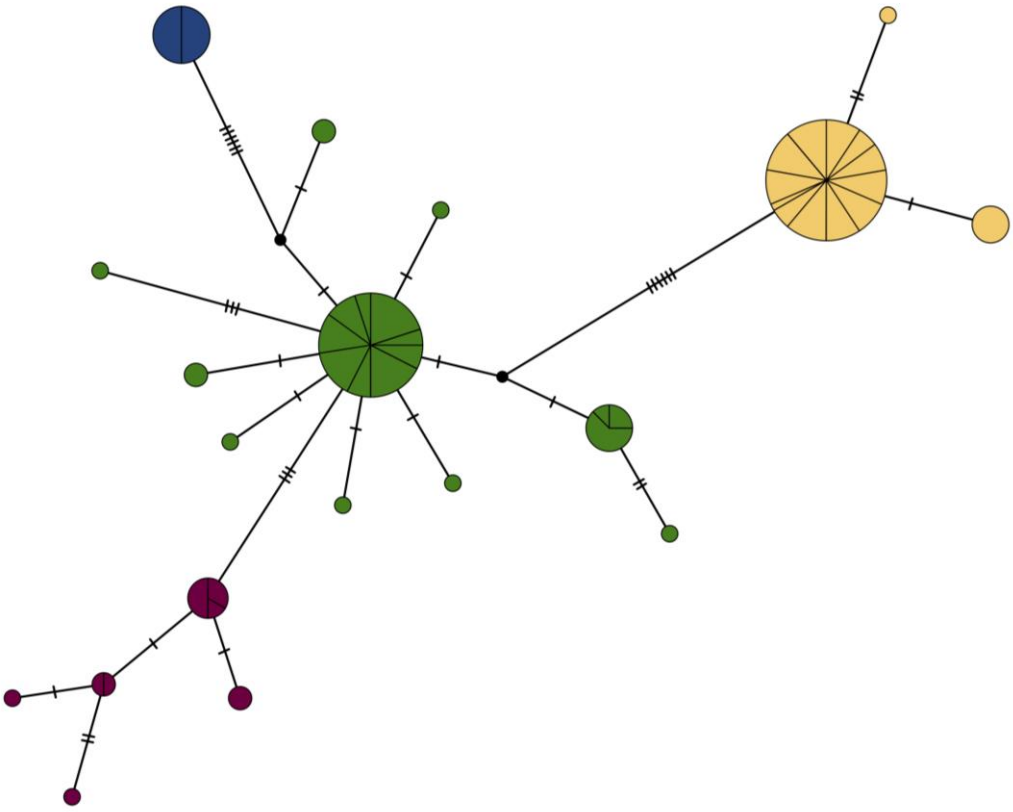
N: number of sequences, S: segregating sites, h: number of haplotypes, Hd: haplotype diversity, *k*: average number of differences, π: Nucleotide diversity, T's D: Tajima's D, FFs: Fu's Fs statistic

*Significant value P<0.05

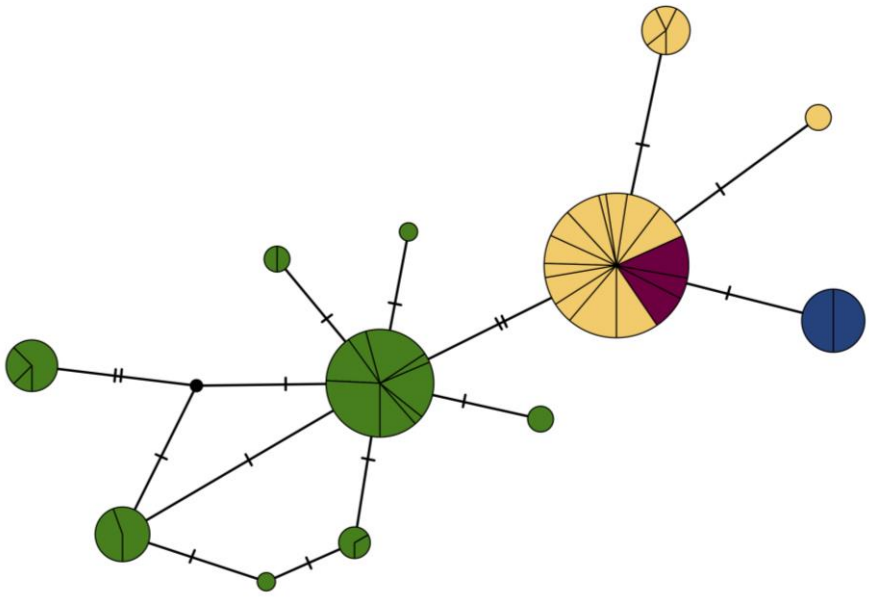
Table 3. Estimates of evolutionary divergence and pairwise Φ_{ST} genetic distances for CytB and 16S lineage

	ATL	**CHI**	**VER**	**PAC**
ATL	---	0.749***	0.818***	0.716***
CHI	0.73***	---	1.00***	0.086***
VER	0.93***	0.93***	---	0.877***
PAC	0.91***	0.91***	0.99***	---

Pairwise Φ_{ST} values for 16S are showed above the diagonal and for CytB below the diagonal. p-value *** <0.001



a.



b.

Figure 2. Median-joining networks for a. CytB and b. 16S lineages for *Agalychnis moreletii*. Circle size represents the haplotypes frequencies. The number of mutations between haplotypes are showed with cross marks. Different colours highlight the different clusters – ATL (green), PAC (yellow), VER (blue) and CHI (maroon).

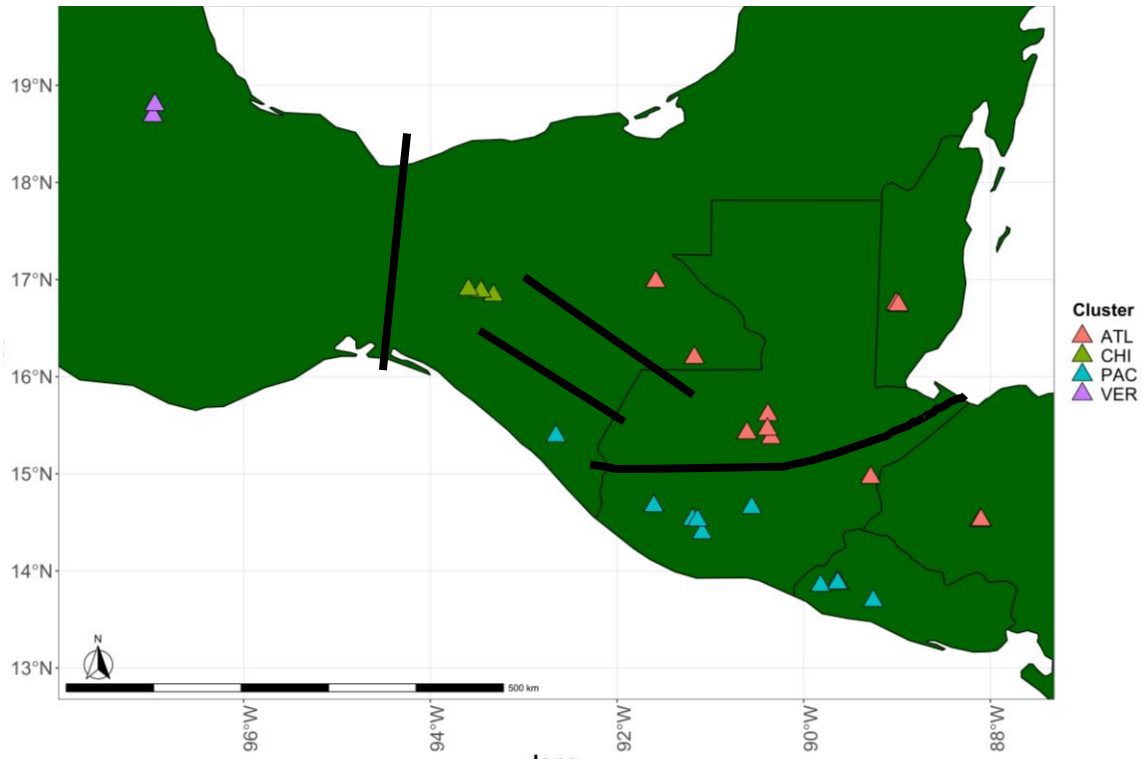


Figure 3. Map showing the sampling sites and highlighting the 4 identified clusters. Lines indicate the biogeographical barriers between them: Tehuantepec Isthmus, Sierra Madre de Chiapas, Chiapan-Guatemalan highlands and Motagua-Polochi Fault Zone.

Phylogenetic analysis

The final data set for the construction of the phylogenetic tree included 129 aligned sequences of 1245 nucleotide positions in length. The mutational model GTR + I + G was used for all the data sets. Phylogenetic analyses based on BI and ML methods showed similar tree topologies (Figure 4). Based on our results we inferred four main clades with high levels of support: Atlantic coast populations (ATL), Pacific Coast populations (PAC), eastern Chiapas populations (CHI) and Veracruz populations (VER) (Figures 2 and 3). This is the same pattern found with the haplotype network analysis (Figure 1) and similar results were found using microsatellite markers (chapter 1).

Figure 4. Phylogenetic reconstruction of *A. moreletii* relationships between populations using concatenated alignments of CytB and 16S. Numbers on branches indicate the posterior probabilities from the Bayesian analysis and percentages of bootstrap values from ML analysis. Colours indicate the different lineages ATL (green), PAC (yellow), CHI (maroon), VER (blue) lineages.

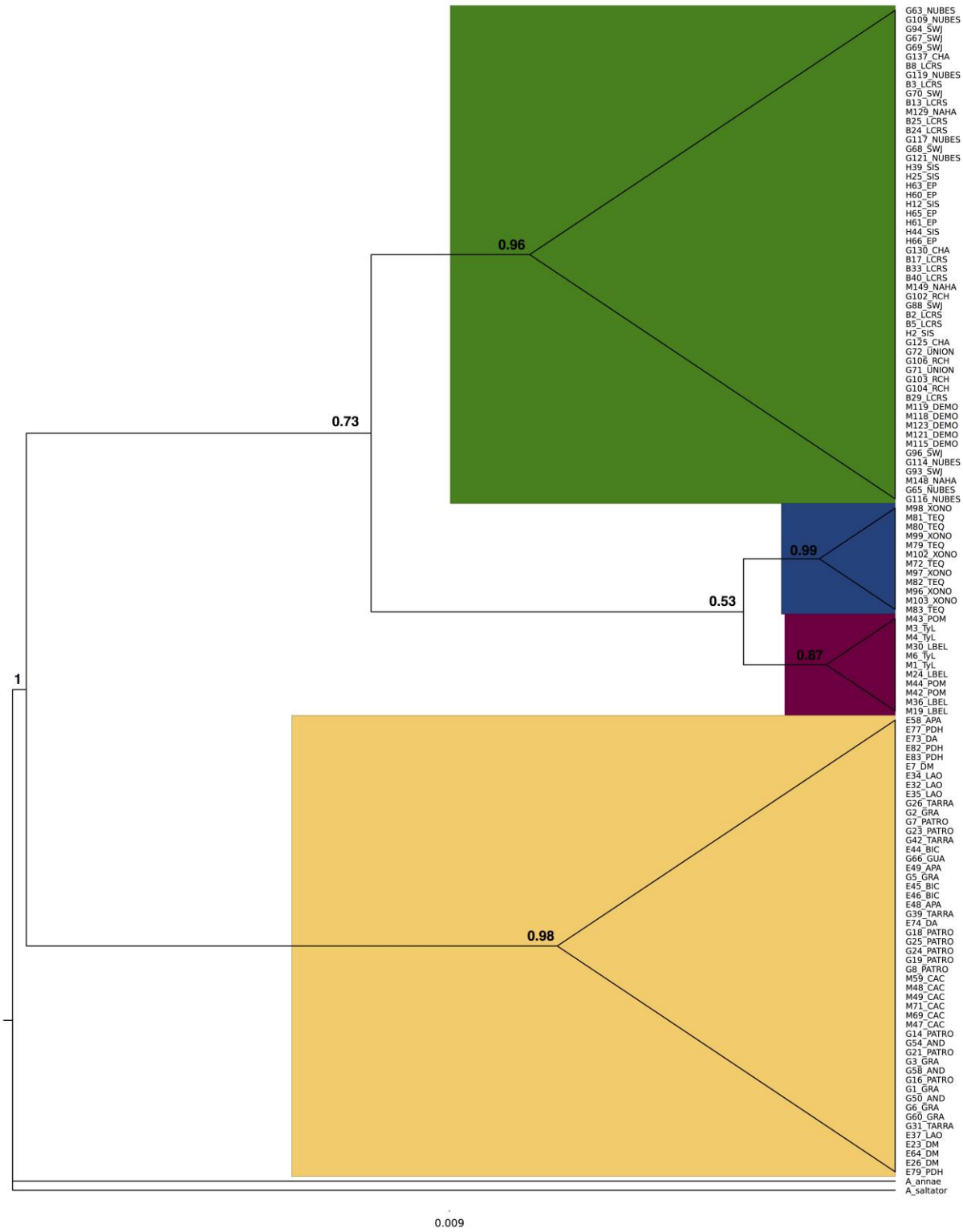


Figure 5. Phylogenetic reconstruction of *A. moreletii* relationships inferred using the Neighbor-Joining method. Numbers on branches indicate the bootstrap values from NJ analysis. Colours indicate the different lineages ATL (green), PAC (yellow), CHI (maroon), VER (blue) lineages.

Discussion

For this study we obtained mtDNA sequences from *Agalychnis moreletii* across its distribution range to determine the genetic diversity and phylogenetic history. Four different clusters of *A. moreletii* were identified: Atlantic coast (ATL) which corresponds to populations from the Lacandon Jungle in Mexico, Northern Guatemala forests, Las Cuevas in Belize and San Isidro in Honduras; Pacific coast populations (PAC) which includes all the populations from El Salvador, the southern coast of Guatemala and southwest Chiapas in Mexico; Eastern Chiapas populations (CHI) and Veracruz populations (VER) (Figure 3).

Results for Tajima's D, Fu's Fs and Fu and Li's F were non-significant when all the individuals were included. When individual clusters were analyzed only ATL cluster showed negative significant results for CytB, which is evidence of rare haplotypes and recent population expansion. The later could be after a bottle neck event, which has been tested using microsatellite markers with significant results for LCRS, LU and NUB populations (chapter 1). LCRS populations seem to have recovered from this event mainly due to the good habitat quality of the reserve. Unfortunately, LU and NUB are populations inhabiting highly degraded forests and the genetic diversity is low.

Genetic differentiation Φ_{ST} values showed high genetic differentiation between all the clusters, which indicated that there is no gene flow among them. Most haplotypes found were exclusive in one of the clusters. It is believed that the absence of haplotypes that are widely distributed across *A. moreletii* range and the high Φ_{ST} values are due to limited connectivity between populations, which has caused them to diverge independently. This could be caused by the isolation of the different clusters due to habitat fragmentation and in some cases different geographical barriers across Mexico and Central America. High differentiation values were also found using microsatellite markers.

The lowest diversity values were observed at the PAC and VER populations. Populations in these areas are greatly affected by habitat fragmentation, which has affected the connectivity and gene flow among populations. In the PAC cluster most of the breeding sites consisted in abandoned wells in farms or pools in back gardens. Most of the sites are surrounded by cane sugar crops that not only have caused habitat destruction but also contaminated water and land (personal obs.). There are some initiatives in Guatemala and El Salvador to protect the frog by the landowners, but there is no real monitoring. VER populations presented the lowest diversity, in general these populations were the most threatened ones. TEQ is a population within a conservation management unit (UMA), but we could not identify any real management or conservation actions to preserve the species, frogs were breeding in small plastic containers set up by the owner and the only habitat for the adults was the backyard with a few trees. The other sampling site (XONO) consisted of a pond next to the main road, without any forest nearby or vegetation cover, this could be the last breeding site on the area. Without any conservation or management plans these populations could disappear in the next decade.

The populations with higher diversity were the ones within CHI and ATL clusters. The ones within CHI have some degree of protection with different management objectives. LBEL is an ecological and educational reserve managed by UNAM University, it used to be a lagoon but due to eutrophication water lillies have fill up the lagoon creating an ideal place for *Agalychnis* frogs. On this site both *A. moreletii* and *A. callidryas* coexist with several other species of amphibians. POM is a pond within the Biosphere Reserve Selva El Ocote, the breeding site is surrounded by forest and there are a lot of conservation actions taking place in this site. The ATL populations are the better preserved along the distribution range of the species. LCRS in Belize and the populations in the Lacandon Jungle in Mexico are pristine forests with a high level of connectivity between them as well as a high degree of protection from governmental agencies. In the case of Guatemala, the species inhabits semi-disturbed sites, some with good management, usually within private lands. Only in SWJ and RCH the frog breeds in natural ponds. However, some habitats within ATL (LU and NUB in Guatemala and SIS and EP in Honduras) are very disturbed usually surrounded by coffee plantations or houses. In the case of Honduras, it was very

difficult to find the species even in the historical distribution sites. Some of the sites were *A. moreletii* is known to inhabit where impossible to visit due to security reasons.

Phylogenetic analysis

The final data set for the phylogenetic tree included 129 samples that amplified for both genes. The GTR+G+I evolutionary substitution model was used to construct the phylogenetic tree. Patterns among populations were very similar as the ones found with microsatellite markers (chapter 1), however four different clusters were identified, splitting the populations of TyL, LBEL and POM from ATL into their own cluster (CHI). Between Mexico and Central America there are several geographical barriers that could have played a role in the diversification of *A. moreletii*. The main barriers are the Tehuantepec Isthmus, Sierra Madre de Chiapas and Chiapan and Guatemalan highlands, the Motagua-Polochic Fault Zone and the Honduras Chortis Block highlands.

The populations from VER are situated North of the Tehuantepec Isthmus which isolates them from all the other populations in this study, all other *A. moreletii* populations North of the Isthmus should be considered different from the populations South of the Isthmus. CHI populations are in a valley, separated by ATL by the Chiapan-Guatemalan highlands and from PAC by the Sierra Madre de Chiapas. The clusters that have showed the highest differentiation levels between them are ATL and PAC, these clusters are divided by the Motagua-Polochic Fault Zone in Guatemala and the Chortis Block that divides the Honduran and El Salvadorian populations. Several species have shown to have similar diversification and dispersal patterns (González-Porter *et al.*, 2013; Suárez-Atilano *et al.*, 2014; Nolasco-Soto *et al.*, 2017; Cano *et al.,* 2018). Isolation by distance model done with microsatellite data indicates that differentiation cannot be explained by distance (IBD) and it is likely that *A. moreletii* is a complex of species.

This study shows why it is important to integrate genetic and ecological data when assessing species to have a more accurate picture of the actual status of the different populations. We are presenting evidence that *A. moreletii* should be considered 4 different ESUs with limited geographical ranges and isolated populations, that are susceptible to population decline and extinction. It is important to prioritize the protection of the remaining populations and reassess its status. It is crucial that every ESU has its own management plan since each area is threatened by different pressures, mainly due to human activity. Creating management plans according to each population or cluster needs will allow to maintain as much genetic diversity as possible. The populations of Veracruz, Honduras, and from the Pacific Coast of Mexico and Guatemala need urgent attention since they are the more threatened sites, the habitat is being destroyed at a fast pace and the genetic diversity is very low and it is important to preserve the genetic information of the populations. In contrast the populations from ATL, showed the highest levels of genetic diversity and connectivity between them. By preserving *A. moreletii* habitat several amphibian species will be under protection and thus the ecosystem services they provide.

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Chapter 4 - Effect of the presence of *Batrachochytrium dendrobatidis* on bacterial communities of the skin of *Agalychnis moreletii* from Guatemala

Introduction

Since the 1990s several threats have caused dramatic declines of amphibian populations around the world. One of the main causes is chytridiomycosis, an infectious disease caused by two fungal pathogens, *Batrachochytrium dendrobatidis* (Bd) (Berger *et al.,* 1998) and *B. salamandrivorans* (Bsal) (Martel *et al.*, 2013). *B. dendrobatidis* has caused the decline and extinction of several populations in Australia and Central America (Blaustein & Kiesecker, 2002; Stuart *et al.* 2004; Lips *et al.* 2005; Mendelson III *et al.* 2006; Wake & Vredenburg, 2008). Mostly chytridiomycosis has affected species associated with water bodies in high altitude cloud forests (Lips *et al.* 2003; Scheele *et al.* 2019), however, there are reports of Bd in lowland tropical species in the Peruvian amazon (Von May *et al.* 2018; Russell *et al.*, 2019). Mesoamerica is one of the most affected regions, where Bd has caused the decline of 228 amphibian species, mostly during the 1990s (Scheele *et al.*, 2019) and 17 species are presumed extinct in the area (Alroy, 2015).

Amphibian skin is an important organ for electrolyte and gas exchange, and it is the first line of defence of these animals. Pathogens like Bd and Bsal affects the keratinized cells in the skin of these animals, consequently scientific efforts have been focused on the study of amphibian's skin composition and the symbiotic relationship that exists between the host and the skin's microbiome (Brucker *et al.* 2008; Brucker *et al.* 2008b; Harris *et al.* 2009; Becker *et al.* 2015). A variety of bacterial communities have been described for several species and ecosystems around the world (Flechas *et al.*, 2012; Kueneman *et al.*, 2014; Becker *et al.*, 2017; Bletz *et al.*, 2017; Hernández-Gómez *et al.*, 2017; Abarca *et al.*, 2018; Griffiths *et al.*, 2018; Ellison *et al.*, 2019b). These bacterial communities produce antifungal

metabolites of which some have the capacity to inhibit Bd infection, playing an important role in the immunity of the host (Rollins-smith & Conlon, 2005; Brucker *et al.*, 2008; Brucker *et al.*, 2008b). The simple presence of the bacteria is not the only factor in successfully inhibiting Bd, density and composition of the bacteria in the skin, as well as the level of infection of the host also play an important role (Harris *et al.*, 2009; Becker *et al.*, 2015).

Amphibian skin bacterial communities can be influenced by various factors in the ecosystem, thus affecting the host's response to disease. Changes in the environment can alter the host microbiome which can have great consequences for the animals health (McCoy & Peralta, 2018). The microhabitat and temperature can influence the structure of the bacterial communities. Varela *et al.*, (2018) found that the pH of the soil has an effect on the microbiome diversity of *Dendrobates auratus* in Panama. In several species it has been recorded that that Bd infection is higher at lower temperatures (Robak & Richards-Zawacki, 2018; Muletz-Wolz *et al.*, 2019).

Different amphibian species within the same environment can present diverse bacterial compositions. Abarca *et al.* (2018) studied several species of amphibians at La Selva Biological Station in Costa Rica and determined that the bacterial composition depended on the host's species or family. Rebollar *et al.* (2016) studied frogs from the same environment in Panama and concluded that the skin bacterial composition is influenced by the host susceptibility to Bd. Host influence on the bacterial communities has been recorded in other tropical (Bletz *et al.*, 2017) and temperate species at different life stages (Mckenzie *et al.*, 2012; Kueneman *et al.*, 2014).

While geographical location can have an impact on the community composition of the same species (Abarca *et al.*, 2018b), temporal variation has also been recorded to have an impact on the alpha and beta diversity in bacterial communities of *Philoria loveridgei* from Australia (Familiar-López *et al.*, 2017) and in *Lithobates yavapaiensis* from Puerto Rico (Longo *et al.*, 2015). Bacterial composition also differs across the

different developmental stages of the individuals which can affect the way they respond to diseases (Kueneman *et al.*, 2014; Griffiths *et al.*, 2018).

B. dendrobatidis infection levels can influence the host's microbiome. Jani & Briggs, (2014) determined in an *ex-situ* experiment that the presence of Bd affects the structure of the bacterial composition in *Rana sierrae*. While Ellison *et al.*, (2019) studied wild populations in California and concluded that higher levels of Bd infection correlated with lower numbers of bacterial alpha and beta diversity. Studies with *P. loveridgei* also documented that higher infection levels correlated with lower relative abundance of OTUS (Familiar-López *et al.*, 2017).

Black-eyed tree frog, *Agalychnis moreletii*, and Batrachochytrium dendrobatidis

Chytridiomycosis has been reported in *Agalychnis moreletii* populations of Belize (Kaiser & Pollinger, 2012) El Salvador (Lawson *et al.*, 2009) and Mexico (Lips *et al.*, 2004; Aanansen 2006). Lips *et al.* (2004) did amphibian surveys in 4 different regions of Mexico and determined that the decline of *A. moreletii* in 2 of these localities was due to Bd, and the species has not been seen in these areas since 1984. Felger *et al*. (2007) found that 98% of the *A. moreletii* tadpoles from several populations of El Salvador presented mouth deformities, due to the pathogen. Lawson *et al.* (2011) reported more populations of *A. moreletii* tadpoles infected with Bd.

In Guatemala the management and conservation of *A. moreletii* varies greatly from site to site. The species can be found in natural ponds within private reserves to abandoned cattle or coffee wells surrounded by plantations or highly fragmented forests. There is no data about the skin microbiome or presence of Bd. Guatemalan amphibians suffered population declines due to infectious diseases in the 1980s and 1990s (Cheng *et al*., 2011). Unfortunately, there is little information about the skin microbiome of the different species and how disease and habitat destruction can impact it.

The aims of this chapter were to describe for the first time the skin microbiome of *Agalychnis moreletii* in sites in Guatemala, as well as to document how it can be influenced by habitat fragmentation and the presence of *B. dendrobatidis*.

Methods

Field surveys

Agalychnis moreletii surveys were done during the rainy season of 2018 in four sites that presented different levels of habitat destruction in Guatemala (Figure 1). The individuals were captured during night hours from 1900-2300 hrs using the visual encounter survey method (VES). All specimens were captured using new sterile gloves and were placed in a plastic bag with a serial number. Data about the microhabitat, time of capture, GPS location and activity of the individual was recorded.

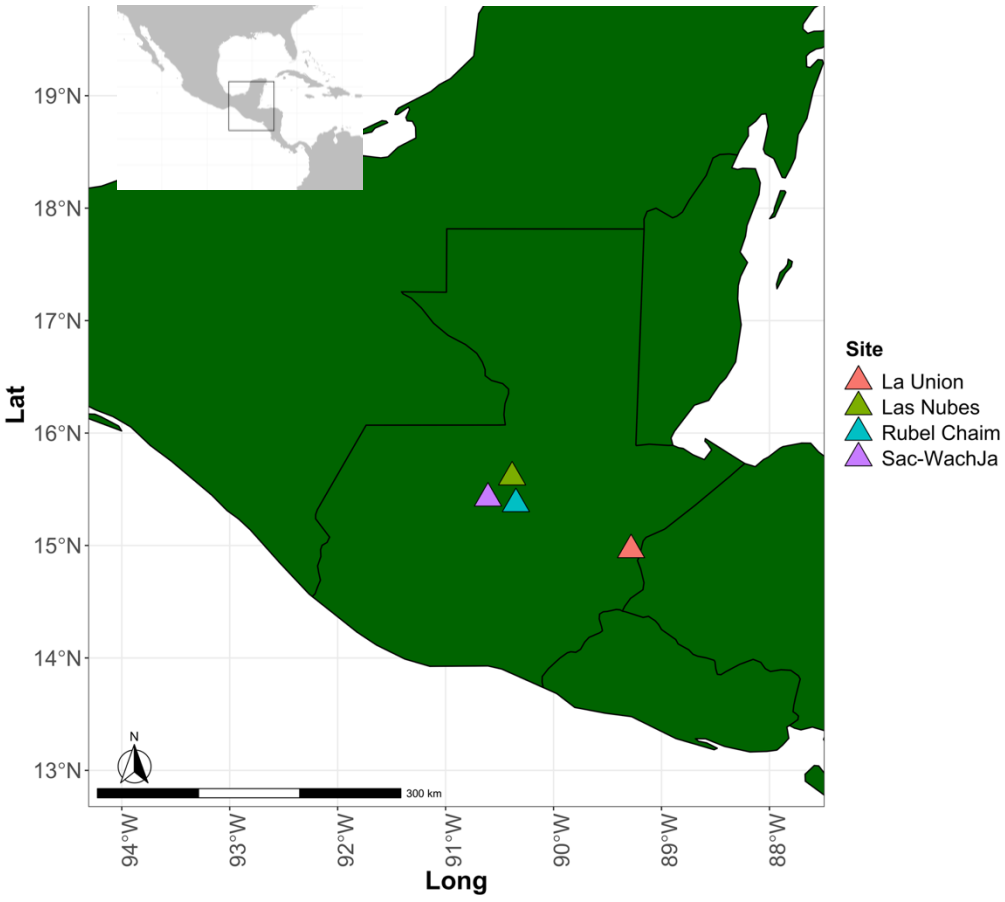


Figure 1. Sites where *A. moreletii* was collected. Finca Dulce Rocío, La Unión, Zacapa (ZAC); Finca Las Nubes, Cobán, Alta Verapaz (NUB); Rubel Chaim, San Juan Chamelco, Alta Verapaz (RCH); Finca Sac-Wach Ja, San Cristobal, Alta Verapaz (SWJ).

Individuals were swabbed immediately after returning to the campsite. All the frogs were rinsed with 100ml of sterile water to remove any transient bacteria. Skin samples were taken using a sterile rayon swab (MW113, Medical Wire Equipment & Co. Ltd). Frogs were swabbed 5 times on each flank, 10 times on the ventral surface, 5 times on each thigh and 5 times on the dorsal surface. The swabs were stored in liquid nitrogen immediately and transported to Universidad del Valle in Guatemala City, where they were stored at -80° until DNA extraction.

DNA extraction and amplification

DNA extractions were done using the Qiagen DNeasy® PowerSoil® Kit, with the following modifications: C1 incubation was done at 65°C for 90 minutes. 50 ul of C6 were added to the filter and incubated for 10 minutes before elusion. Samples were quantified using a nanodrop™ (Thermo Fisher). For all the extraction sets a negative control was also extracted. Microbial community standard from ZymoBIOMICS ™ (D6300) was extracted as a positive control. DNA samples, extraction negatives and positive controls and PCR negatives were randomly assigned in triplicate into wells in 96 well plates. The plates also contained blanks which were wells left completely empty (Supp info, Figure 3).

PCR reactions were done to amplify the V4 region of the 16S gene using the primers 515F (Parada *et al.*, 2016) and 806R (Apprill *et al.*, 2015). Both primers were ordered with different tag combinations, added to the 5' -end of the primers. This allowed us to barcode each PCR product and to identify each sample after the sequencing run (Supp info table 1). The primers were diluted to 100uM and used directly on the master mix. The PCR master mix was done to a final volume of 10ul; using 5ul of AmpliTaq Gold™ 360 Master Mix (applied biosystems), 2 ul of each primer (final concentration 1uM) and 1ul of DNA. The PCR program used was 95°C for 10 minutes, followed by 32 cycles of 95°C for 30 seconds, 50°C for 60 seconds and 72°C for 90 seconds, and a final extension of 72°C for 30 minutes. PCR fragments were visualized

in 1% agarose gels. All the samples, controls and blanks from one plate were pooled into a 1.5ml Eppendorf tube and frozen at -80 until library preparation. Each one of these pools was treated as a single library.

Library preparation

Library preparation was done using Illumina® TruSeq® Nano DNA Library Prep kit. 200ng of each pooled sample was used as DNA input for 350bp insert size. Index adaptors 006, 007 and 012 were used for the different libraries. The quality of the final libraries was checked using a Qubit® dsDNA HS (High Sensitivity) Kit (Life technologies) and an Agilent 7500 Bioanalyzer chip with the High Sensitivity DNA Kit (Agilent Technologies). 1nM of each library was denatured using the NextSeq denature and dilute libraries protocol from Illumina® and pooled together. 10% PhiX DNA spike-in control was added. The library had a final concentration of 18pM. 600 ul were loaded into an Illumina® MiSeq Reagent Kit v2 Nano (2 x 150 bp) flow cell.

Chytridiomycosis testing

Samples were tested for chytrid fungus with real-time PCR (qPCR) to amplify the ITS-1- 5.8S rDNA region of *B. dendrobatidis*, using the primers ITS1-3 and 5.8S (Boyle *et al.*, 2004). PCR master mix consisted in 2.35 ul of dH2O, 10ul of SYBR® Green Master Mix (Sigma-Aldrich), 1ul of each primer, 0.05ul of probe Chytr MGB2 (Boyle *et al.*, 2004) 1.60ul of BSA (50mg/ml dH2O). Samples were run in duplicate. Bd DNA standards containing DNA from 100, 10, 1 and 0.1 genome equivalents (GE) and negative controls were run in triplicate. qPCR conditions were 50C for 2 minutes, 95C for 10 minutes, followed by 60 cycles of 95C for 15 secs and 60C for 1 minute.

Data Analysis

Raw sequences were demultiplexed using the Cutadapt function (Martin, 2014) to obtained a set of demultiplexed fastq files. The R package DADA2 (Callahan *et al.*, 2016) was used to filter, dereplicate and to performed the quality controls of the sequences. The reads were trimmed at 220 and 180 base pair position for the forward and reverse, respectively. We filtered out the reads with more than 2 expected errors for the forward and 5 expected errors for the reverse sequences. Samples were merged, an Amplicon Sequence Variant table (ASV) was constructed and chimeras were removed. To assign taxonomy the sequences were aligned against the 16S reference data base SILVA Small Subunit rRNA 138 (Quast *et al.*, 2013). A phyloseq object was created using the package Phyloseq (McMurdie & Holmes, 2013), including ecological information about each individual. Barplots were created to visualize the microbiome structure and relative abundance of the samples and sites, as well as the diversity between infected and non-infected frogs. To visualize the most abundant families of bacteria present in the data set a heat map was plotted.

Alpha diversity was calculated using Shannon and Simpson indices on Phyloseq. To compare the diversity among sites and between Bd positive and negative frogs, Kruskal-Wallis tests were performed. Beta diversity analysis were obtained using weighted and unweighted UniFrac distances. The weighted unifrac considers the relative abundance and the unweighted only takes into consideration presence/absence of the taxa. The Jaccard index was also calculated. The results were visualized using a non-metric multidimensional scaling (NMDS). We performed an analysis of similarity (ANOSIM) and a permutational multivariate analysis of variance (PERMANOVA) using VEGAN (Dixon, 2003) to determine if there was any significant difference among beta diversity and the sampling sites. Additionally, a PERMANOVA was done to determine the effect of Bd positive frogs on the bacterial diversity.

Finally, the ASV table was compared against the Antifungal Isolates Database (Woodhams *et al.*, 2015) using Geneious Prime ® 2019.2.1 (Biomatters Ltd). We

retain the reads that had shown to be potentially Bd inhibitors and that had a match of at least >97% with the bacterial sequences from the database. Inhibitory bacterial isolates were compared against Bd infected frogs to determine if there was any relationship between them.

B. dendrobatidis analysis

Infection intensity was defined as the number of zoospore equivalents ZE (Vredenburg *et al.*, 2010). These were calculated by multiplying by 20 the quantity values obtained in the output file from the qPCR (Bletz *et al.*, 2015) to remove the dilution factor from the extractions. A sample was considered positive for Bd when the ZE was ≥1 and negative when the ZE was <1.

Results

We identified 1233 ASVs from *Agalychnis moreletii* samples that belong to 21 phyla being the most abundant across the sites Proteobacteria and Actinobacteriota (Figure 3). At the genus level we identified 86 orders being the most prominent Burkholderiales, Micrococcales and Pseudomonadales. Of the 116 bacteria families identified in the data set, Alcaligenaceae and Pseudomonadacea were the most abundant (Figure 4), the core microbiome was composed mainly of ASVs assigned to the Alcaligenaceae family and the *Verticiella* genus (Supp info, Figure 1,).

Figure 3. Phyla abundance by sample and grouped by site. Proteobacteria was the most abundant Phylum occurring across all the sites. Abbreviations of sites names: La Unión, Zacapa (ZAC); Finca Las Nubes, Cobán, Alta Verapaz (NUB); Rubel Chaim, Alta Verapaz (RCH); Finca Sac-Wach Ja, San Cristobal, Alta Verapaz (SWJ).

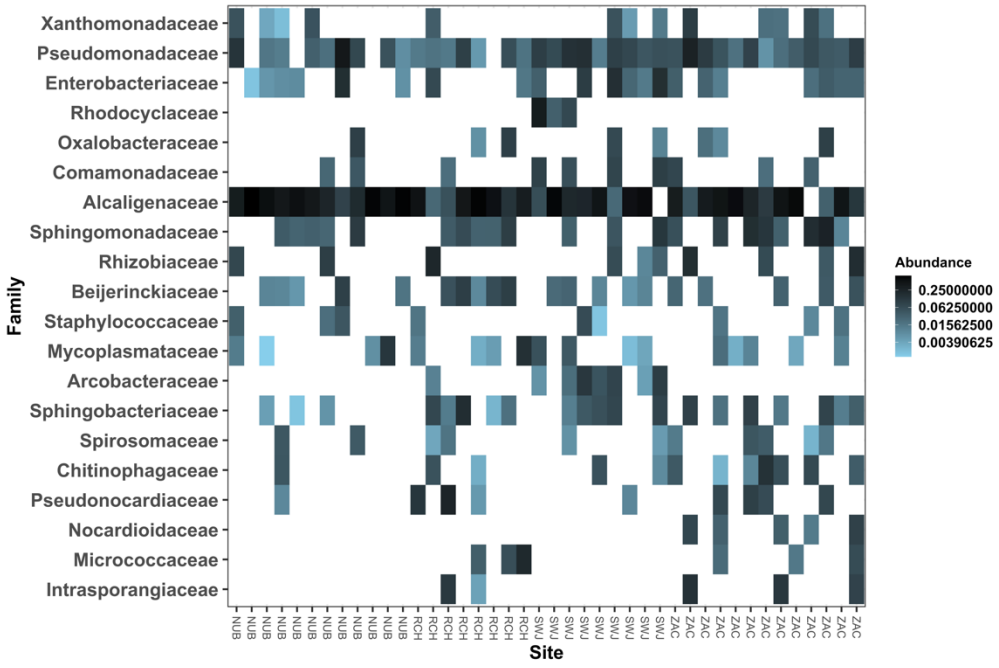
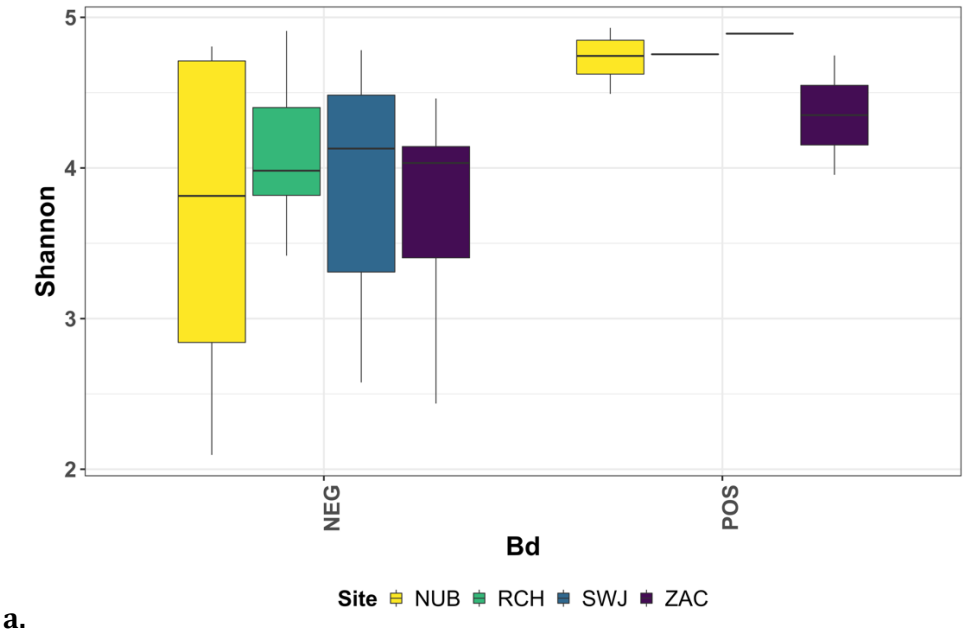


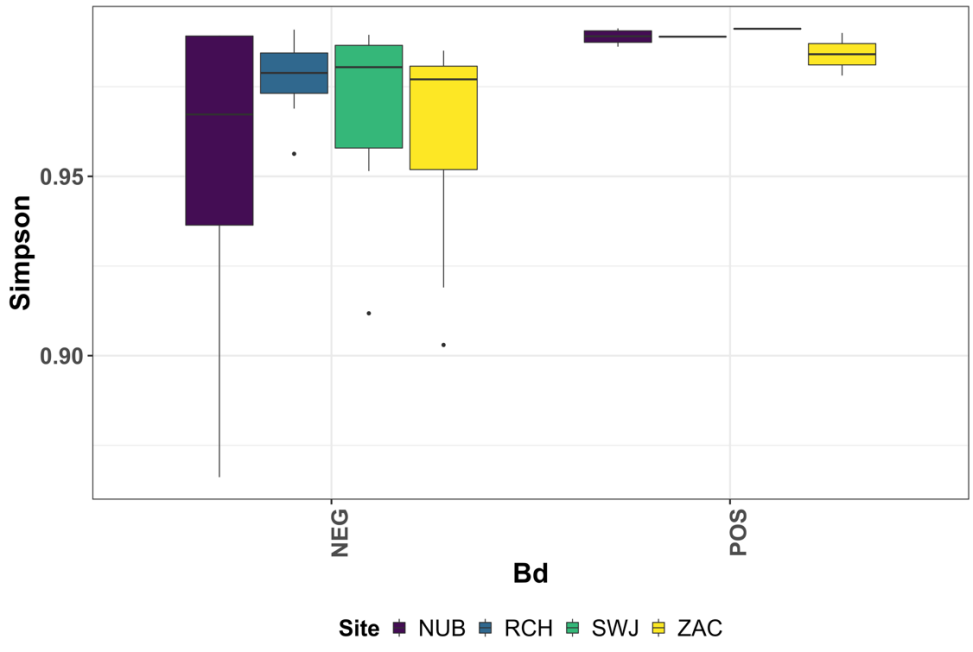
Figure 4. Heat map showing the 20 most abundant families (ASVs). Alcaligenanceae and Pseudomonadacea were the most abundant families across all sites.

Alpha and Beta Diversity

The lowest levels of alpha diversity were found in the population NUB (Figure 5). On this site frogs inhabit old cattle tanks around the main house of the farm and there is not much forest cover surrounding the area. *A. moreletii* skin bacterial diversity was very homogeneous, when comparing the alpha diversity among the 4 sites we could not find a significant difference between sites for richness (K-W chi-squared = 2.53; df= 3; p-value = 0.47) or evenness (K-W chi-squared = 2.83; df= 3; p-value= 0.42). For beta diversity we did not find any clear pattern in terms of the bacterial composition when calculating weighted Unifrac distances (PseudoF=1.78, R^2=0.12, p-value=0.042) or Jaccard index (PseudoF=1.07, R^2=0.08, p-value= 0.26) (Figure 6). The unweighted Unifrac distances did show significant differences in the bacterial community composition among sites (PseudoF=1.54, R^2=0.11, p-value= 0.010), which demonstrates that the difference is due to the presence or absence of certain groups among the sites.

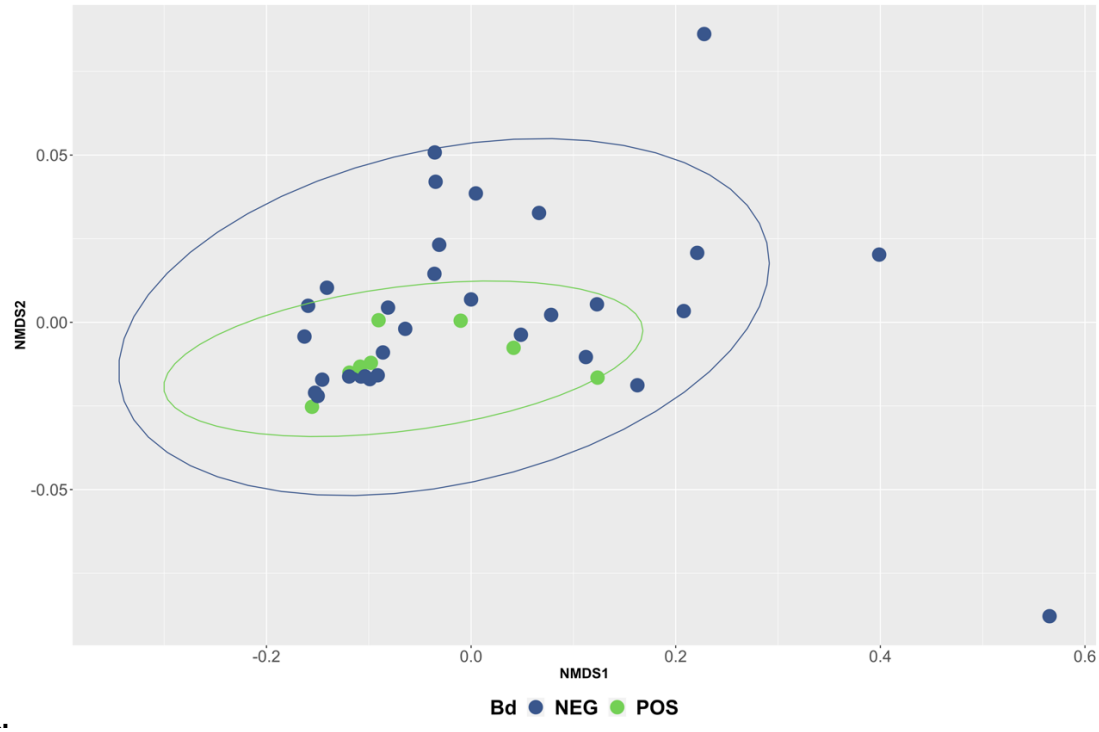


a.

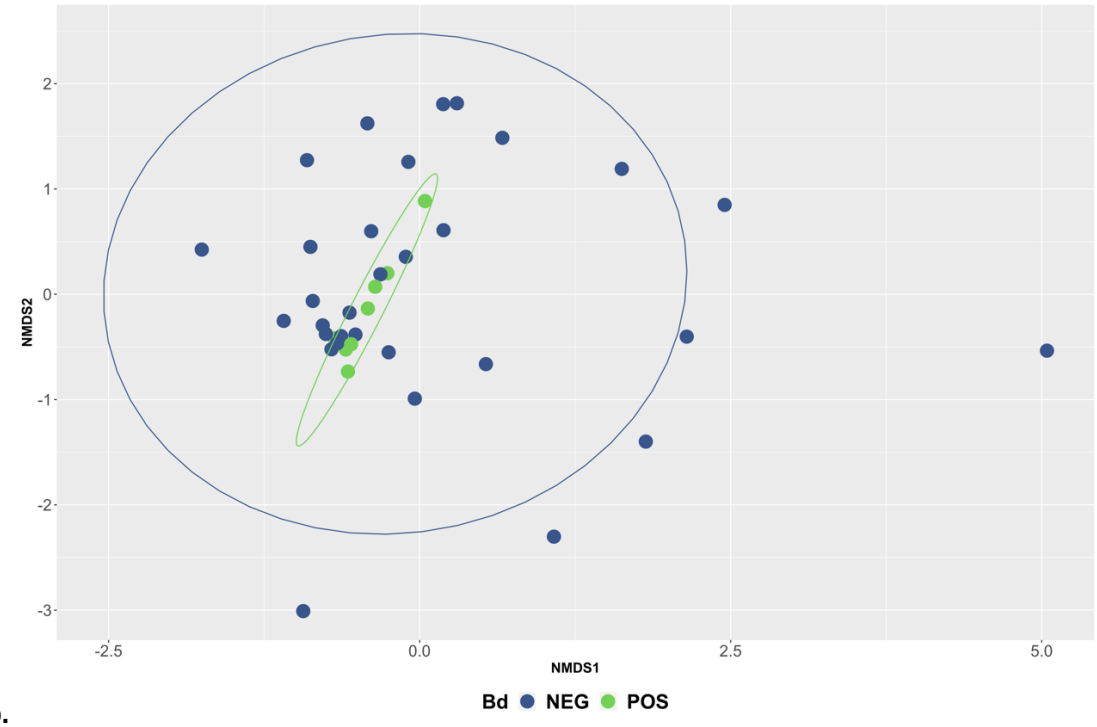


b.

Figure 5. a. Shannon and **b.** Simpson indices for the 4 sampling sites, showing the differences between Bd positive and Bd negative individuals. Line in boxes represent the median, whiskers (vertical lines) represent the maximum and minimum values, boxes go from the first quartile to the third quartile.



a.



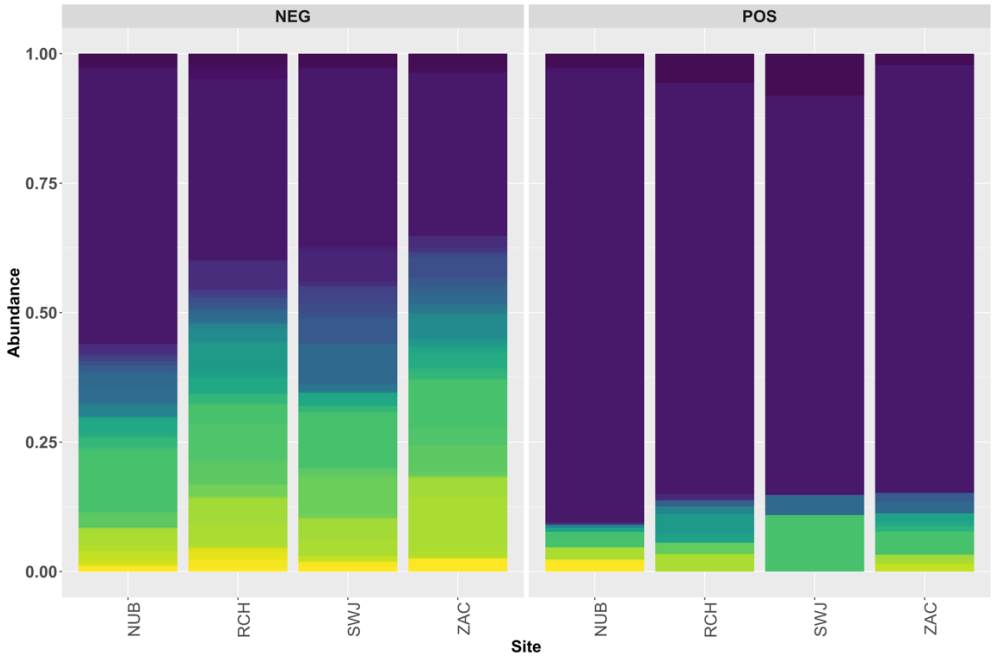
b.

Figure 6. NMDS of Beta diversity indices of **a.** weighted Unifrac distances **b.** Jaccard index. In both graphs positive Bd clustered tighter than negative Bd samples. Showing that the bacterial community structure could be defined by the infectious status of the individuals.

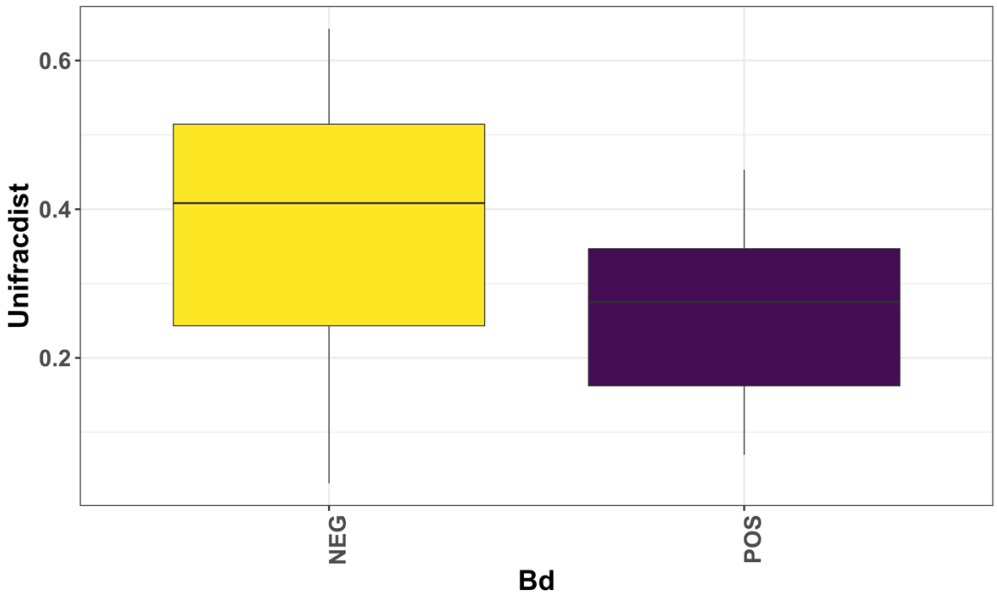
Of the 42 samples only 8 were Bd positive, 4 from NUB, 2 from ZAC and 1 from RCH and SWJ. Infection load was low for all the positive samples. In the field we did not detect any signs of lethargy or infection in any of the positive frogs. The relative abundance barplots (Figure 7) showed clear differences in the bacterial community composition between infected and non-infected frogs. The community structure of the non-infected frogs was dominated by the genus *Verticiella* followed by *Pseudomonas,* while the infected frogs' bacterial composition was comprised mainly of *Verticiella.*

When testing the effects of Bd on the bacterial communities, we found that alpha diversity was significantly lower in Bd positive frogs (Figure 5), for both Shannon and Simpson indices (Shannon: W = 24, p-value <0.001; Simpson: W = 28, p-value< 0.001). Beta diversity was also significantly lower between in infected frogs for the Jaccard index and weighted Unifrac distances (PseudoF=1.922, R^2= 0.05, p-value= 0.008; PseudoF= 3.02, R^2= 0.07, p-value= 0.02). While for the unweighted Unifrac there was no significant difference between the bacterial communities (PseudoF=1.21, R^2= 0.33, p-value= 0.2012), indicating that the differences among the communities were due to differences in ASVs relative abundance instead of presence or absence of the ASVs.

In total we found 90 ASVs that corresponded to bacteria with potential Bd inhibitory characteristics. All the samples contained potentially anti-Bd bacteria isolates, which corresponds to 7% of the ASVs in our data set. There was no significant relationship between potentially inhibitory bacteria (K-W chi-squared = 1.76; df= 3; p-value = 0.62) and the presence of Bd.



a.



b.

Figure 7. a. Bacteria family abundance in frogs with Bd and without Bd infection. **b.** Unifrac distances comparing infected and non-infected frogs. Diversity was lower on Bd infected amphibians.

Discussion

For this project we study the skin-bacterial communities and Bd presence in *Agalychnis moreletii* from 4 sites of Guatemala. This is the first study to characterize the bacterial communities on the skin of the species. Proteobacteria was the most abundant Phylum across all the sites, as well as the orders Burkholderiales, Micrococcales and Pseudomonadales, similar to what previous studies have described in other tropical species (Rebollar *et al.*, 2016; Becker *et al.*, 2017; Abarca *et al.*, 2018b; Varela *et al.*, 2018). Of all the identified bacterial families Alcaligenaceae was the most abundant, this family has also been recorded as the most prominent one in *A. callidryas* from different environments in Costa Rica and Panama (Belden *et al.*, 2015; Abarca *et al.*, 2018).

This pattern could imply that in *Agalychnis* frogs the bacterial community composition is associated mainly to the host instead of the environment as has been recorded for several species around the world (Mckenzie *et al.* 2012; Kueneman *et al.*, 2014; Abarca, *et al.*, 2018; Varela *et al.*, 2018). Similar to our results, Bletz *et al.* (2017) found that in arboreal species from Madagascar, Alcaligenaceae is also the most abundant family across different sites and seasons, implying that the bacterial composition of the black-eyed tree frog could be influenced by its arboreal microhabitat and the abiotic factors that affect it.

When comparing the bacterial diversity among the four sampling sites we did not find a significant difference between the sites. Even if the environment and the breeding sites where the frogs were collected are very different. While there were no significant differences among sites, NUB showed the lowest levels of alpha diversity. This farm is an old coffee plantation in the North of Guatemala, there is not much of the original forest left and the site is surrounded by palm oil and coffee plantations. The individuals of *A. moreletii* can be found breeding in old cattle or coffee wells without any plant cover to protecting them. This can have an impact on the diversity of the bacterial communities and how they respond to potential pathogens. Michaels *et al.* (2014) found that when *A. callidryas* is kept in captive

conditions without plant cover the abundance and richness of the bacterial species declines significantly, which can leave the individuals susceptible to pathogens.

Only 8 frogs were positive for *B. dendrobatidis* and all the infection levels were low across sites. Our results showed a significant reduction between the richness and evenness as well as changes in the bacterial community composition in Bd positive frogs. While the bacteria composition in non-infected frogs was comprised of a larger variety of taxa; the bacterial communities of the infected frogs were dominated by the orders Burkholderiales and Micrococcales; the Alcaligenaceae family and the *Verticiella* genus (Supp info Figures 1, 2). Bacteria from the orders Burkholderiales and Micrococcales have been identified as potential Bd inhibitors (Woodhams *et al.* 2015), the higher abundance of these two orders could be a response to the presence of the pathogen aiding the host to fight the infection and avoiding its death. Even if the frogs were infected, we did not see any signs of lethargy, abnormal postures or lack of reflex (Berger *et al.* 2005) as it has been documented for other species. NUB population presented the highest number of infected individuals as discussed above this could be related to a degraded environment which can cause a decline in the bacterial composition.

Beta diversity and community structure was also significantly lower among infected frogs. A decline in bacterial diversity when frogs are infected with Bd has been recorded in species from different environments (López *et al.*, 2017; Ellison *et al.*, 2019). When visualizing the beta diversity using a NMDS we find that Bd positive samples tend to cluster tighter than samples from Bd negative individuals. Showing that the infectious status defines the variation in the bacterial community structure.

We could assign 90 ASVs (7.30%) to one of the bacterial isolates on the Bd database of (Woodhams *et al.*, 2015), indicating that *A. moreletii* skin-bacterial communities can potentially protect them from Bd. There was no significant difference between the Bd positive or negative frogs and the number of potential Bd-inhibitors. Even if the skin bacterial composition of this species carries potentially anti-Bd bacteria it is important to keep monitoring the populations for any change. It is known that low

bacterial diversity might provide Bd an opportunity to colonize and cause the death of the host (Jani & Briggs, 2014).

B. dendrobatidis has caused the declined of several species around the world, it is important to keep doing surveys in the region, since Mesoamerica has been one of the most devastated regions by the pathogen (Scheele *et al.*, 2019). Bd caused the decline of *A. moreletii* populations in Mexico (Lips *et al.*, 2004) and it has been detected in several populations of El Salvador and Belize (Lawson *et al.*, 2009; Kaiser & Pollinger, 2012), which shows that it is widespread along the distribution range of the species. This is the first time the disease is detected in Guatemala. The lower diversity of bacterial communities documented in Bd infected frogs could lead to future health problems for the populations, and it is important to do more surveys in different sites.

Further studies characterizing *A. moreletii* ecosystem microbiome are needed to determine if the community composition is influenced only by the host or if the environment also plays a role in it. *Ex-situ* studies with controlled variables could be beneficial to understand the dynamics of skin associated bacteria, the environment and infectious diseases in *Agalychnis* frogs, especially since some species of the genus are catalogued as critically endangered by the IUCN Red List. Understanding the host-environment-microbiome complex in amphibians is important for the health of the populations and should be taken into consideration when assessing the different species or during the creation of conservation plans (e.g. reintroductions and translocations).

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Supplementary information

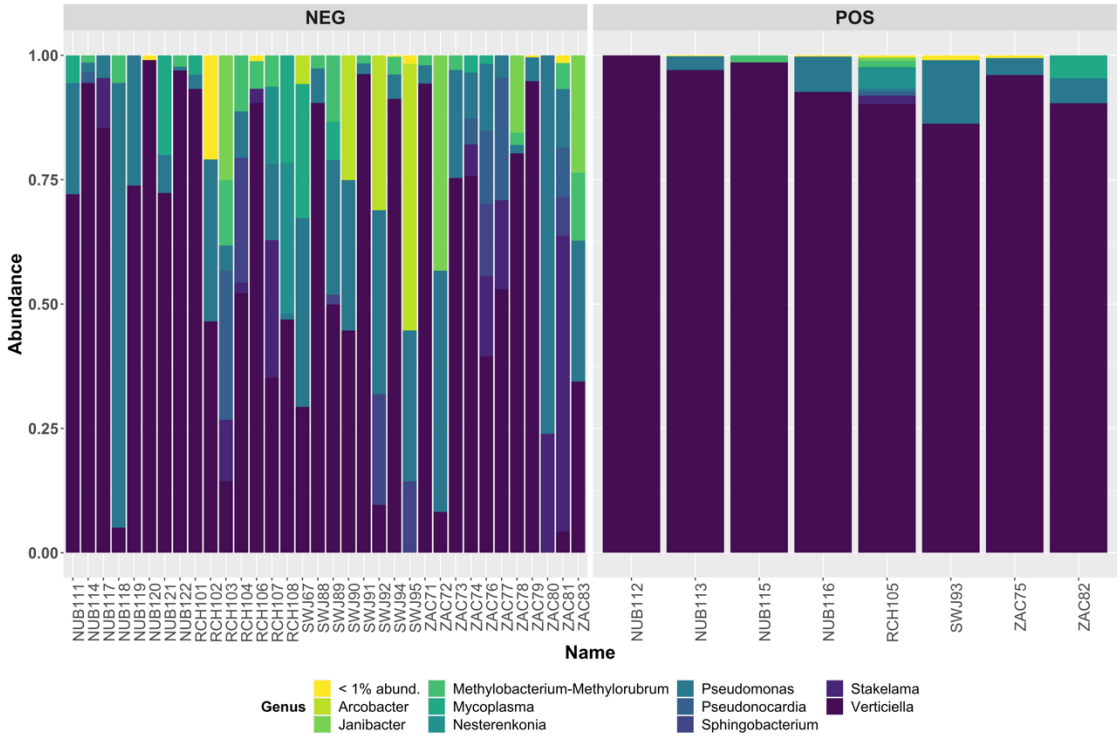


Figure 1. Top 10 genus identified in Bd negative and positive individuals of A. moreletii.

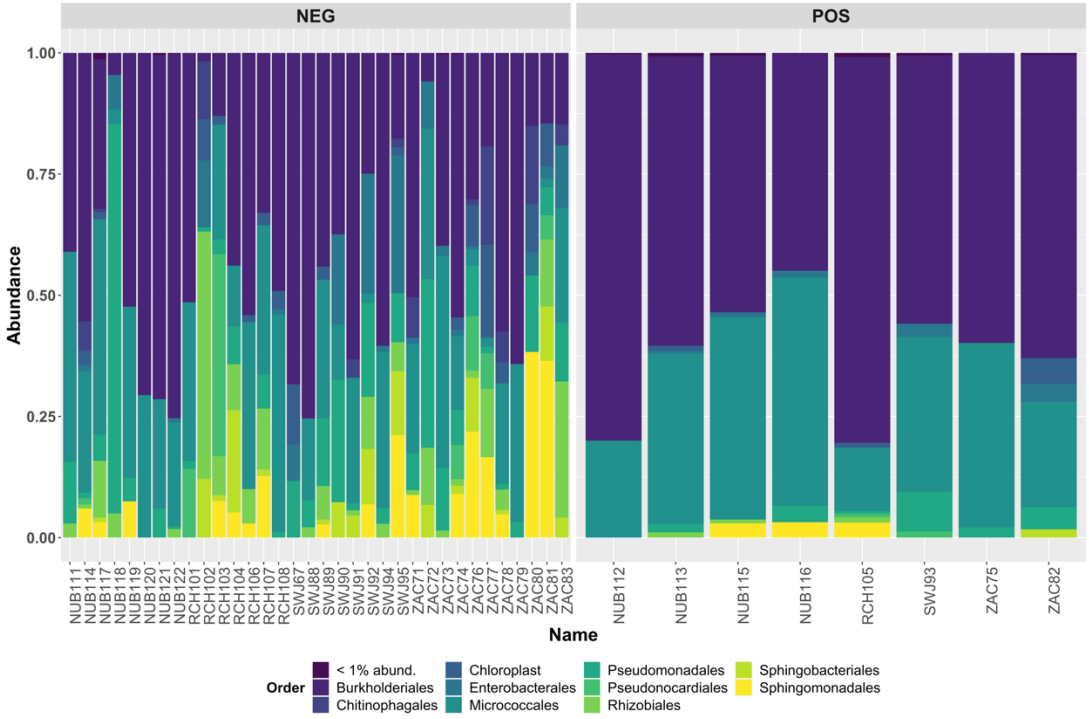


Figure 2. Top 10 orders identified in Bd negative and positive individuals of A. moreletii.

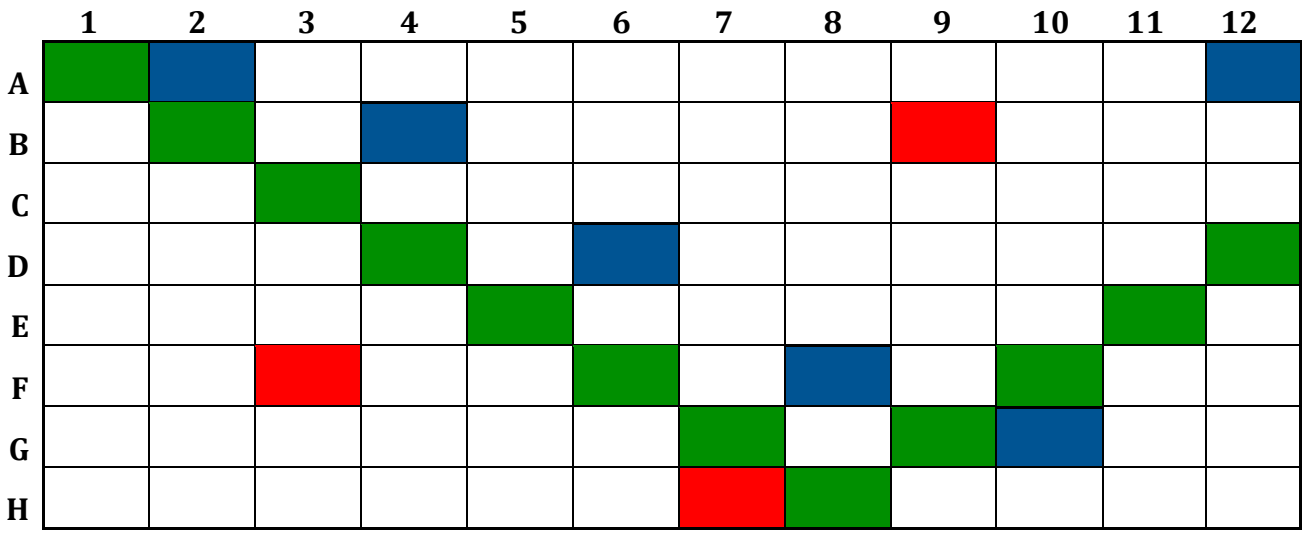


Figure 3. Example of a plate template to randomize the samples for sequencing. Blue wells: positive controls; red wells: PCR negatives; green wells: blanks.

Table 1. Phasing and Barcodes used to tag the Forward and Reverse primers (Taberlet *et al.* 2018)

Name	Phasing	BARCODE	Name	Phasing	BARCODE
1FWD	NN	acacacac	15REV	NNN	tgacatca
2FWD	NNNN	acagcaca	16REV	NN	acatgtgt
3FWD	NNN	gtgtacat	17REV	NNNN	gtacgact
4FWD	NN	tatgtcag	18REV	NNN	atgatcgc
5FWD	NNNN	tagtcgca	19REV	NN	acgacgag
5FWD	NNN	tactatac	20REV	NNNN	catcagtc
7FWD	NN	actagatc	21REV	NNN	atcagtca
8FWD	NNNN	gatcgcga	22REV	NN	tctactga
1REV	NN	acacacac	23REV	NNNN	gatgatct
2REV	NNNN	acagcaca	24REV	NNN	ctgcgtac
3REV	NNN	gtgtacat	25REV	NN	agcgacta
4REV	NN	tatgtcag	26REV	NNNN	tcagtgtc
5REV	NNNN	tagtcgca	27REV	NNN	actctgct
5REV	NNN	tactatac	28REV	NN	atatagcg
7REV	NN	actagatc	29REV	NNNN	ctatgcta
8REV	NNNN	gatcgcga	30REV	NNN	tcgcgctg
9REV	NNN	cgctctcg	31REV	NN	agcacagt
10REV	NN	gtcgtaga	32REV	NNNN	tagctagt
11REV	NNNN	gtcacgtc	33REV	NNN	agtgctac
12REV	NNN	gactgatg	34REV	NN	cgtataca
13REV	NN	agactatg	35REV	NNNN	cgagtcgt
14REV	NNNN	gcgtcagc	36REV	NNN	cacatgat

Chapter 5 - Amphibian skin microbiome changes across husbandry phases, a case study with *Plectrohyla matudai* and *Bolitoglossa* spp.

Introduction

In the last decade several threats have been identified as the cause of amphibian declines. One of the main causes are the infectious diseases caused by fungal pathogens, *Batrachochytrium dendrobatidis* (Bd) (Longcore *et al.*, 1999) and *B. salamandrivorans* (Bsal) (Martel *et al.*, 2013). During the last decade researchers have characterized the skin microbiome of several species of amphibians across different ecosystems in order to understand the symbiotic relationship that exists between the host and the skin microbiome (Bletz *et al.*, 2017; Abarca *et al.*, 2018; Griffiths *et al.*, 2018; Ellison *et al.*, 2019; Jiménez *et al.*, 2019).

The amphibian microbiome plays an important part in the immunity and resistance as it is the first line of defense against pathogens (Rollins-Smith & Conlon, 2005; Brucker, *et al.*, 2008). Several bacteria have been identified in multiple host species as Bd inhibitors in locations around the world (Woodhams *et al.*, 2015). Metabolites secreted by these bacteria are an important part of the host's immunity. For example, it has been documented that Violacen and indole-3 carboxaldehyde produced by the bacteria *Janthinbacterium lividum* and 2,4-diacetylphloroglucinol produced by *Lysobacter gummosus* are associated with resistance to *B. dendrobatidis* in amphibians (Brucker *et al.*, 2008; Becker *et al.*, 2009; Harris *et al.*, 2009).

Threats like Bd and Bsal are very difficult to manage *in-situ* and conservation strategies have been developed to attempt to protect wild populations (Browne *et al.*, 2011). Difficulties in *in situ* management of disease has led to the establishment of *ex-situ* studiesof the impacts these diseases have on the host and have accelerated attempts to breed different species with the aim of reintroducing them to the wild (Gagliardo *et al.*, 2008).

It is known that the diversity of bacterial and fungal communities can change when animals are in captivity. These changes, in turn, can have an impact on host mediated immunity (Antwis *et al.*, 2014; Loudon *et al.*, 2014; Sabino-Pinto *et al.*, 2016; Passos *et al.*, 2018; Bates *et al.*, 2019). However, it is not known if this change is due to an alteration in the environment, if it varies among populations or both. Understanding this process and determining how it can impact the health of captive amphibians is essential for husbandry and re-introduction programs.

Taking amphibians from the wild into captivity can be very stressful for the individuals and can cause changes of the associated microbiome communities. Knowing the composition of the skin microbiome is an important aspect in understanding how the animals respond to diseases and stress (Loudon *et al.* 2014) and is essential if we are to understand how captivity alters these communities. Bates *et al.* (2019) demonstrated that captivity has a significant effect on the bacterial and fungal communities of *Lissotriton vulgaris* and *Triturus cristatus.* Husbandry practices can also influence the bacterial community composition in captive individuals. Antwis *et al.*, (2014) found that individuals of *Agalychnis callidryas* that have been fed an enriched carotenoid diet had higher abundance of bacterial communities. Loudon *et al.* (2014) found that the skin microbiome diversity of *Plethodon cinereus* decreases when salamanders are kept under sterile conditions, while the bacterial diversity was retained when salamanders were kept with substrate from the sampling site. These types of experiments are very important to assure the health of the individuals, especially if the aim of the project is to reintroduce the animals into the wild (Loudon *et al.*, 2014).

Ex-situ amphibian research in Guatemala

In Central America, bacterial community studies have focussed on Panamanian (Woodhams *et al.* 2006; Becker *et al.* 2014; Becker *et al.* 2015; Becker *et al.* 2015b), Costa Rican (Abarca, Vargas, *et al.*, 2018; Abarca, Zuniga, *et al.*, 2018; Jiménez *et al.*, 2019) and Belizean species (Antwis *et al.* 2014). It is important to investigate and characterize the bacterial isolates from other species and countries (Harris *et al.* 2009) to understand the dynamics of this symbiotic relationship, especially in sites where *B. dendrobatidis* is present.

Amphibian research in Guatemala has focused on *in-situ* conservation and there is very limited knowledge about *ex-situ* husbandry of local species. No captive programs are known for *Plectrohyla* and only one is known for *Bolitoglossa* at Toledo Zoo, Ohio (pers comm Tim Herman). The IUCN Red List has classified *B. conanti, B. nympha* as endangered and *P. matudai* as vulnerable. Generating information about their biology is fundamental to develop conservation strategies tailored to their needs. *P. matudai* was recognized as good surrogate for *ex-situ* research by Amphibian Ark (Guatemalan conservation needs assessment workshop, 2010) and it has been documented that the species is resistant to Bd (Cheng *et al.*, 2011), thus this is the ideal starting point for *ex-situ* conservation of amphibians in Guatemala.

The aims of this chapter were to characterize the skin microbiome of *Bolitoglossa* spp. and *Plectrohyla matudai* from La Unión, Zacapa in Guatemala; and to examine how it changes once individuals are move from the wild into captivity. We also examined the consequences of husbandry practices on the health of individuals brought into captivity.

Methods

Amphibians were collected in two localities of La Unión, Zacapa in Guatemala, The Municipal Forest adjacent to Finca Dulce Rocio and the forest within the property of Finca La Marsella. Individuals were located using the Visual Encounter Surveys technique (VES) from 2000-2300 hrs during the rainy season of 2018. Swabs were taken immediately after returning to the campsite. Individuals were rinsed with 100ml of sterile water to remove any transient bacteria and skin samples were taken using a sterile rayon swab (MW113, Medical Wire Equipment & Co. Ltd). Frogs were swabbed 5 times on each flank, 10 times on the ventral surface, 5 times on each thigh and 5 times on the dorsal surface. Salamanders were swabbed 10 times on their flanks, the ventral and dorsal surface. Amphibian skin samples were obtained from 37 individuals from 7 species. 3 *Bolitoglossa conanti*, 2 *B. dofleini*, 1 *B. mexicana*, 5 *B. nympha*, 2 *Bromeliohyla*, 2 *Ecnomiohyla* and 22 *P. matudai*. Swabs were stored in liquid nitrogen straightaway and transported to Universidad del Valle (UVG) in Guatemala City where they were stored at -80° until DNA extraction. All the animals were transported to UVG's facility with the exception of the *B. dofleini, Bromeliohyla* and *Ecnomiohyla.*

Figure 1. Maps showing a. La Union Municipal Forest location in eastern Guatemala and b. Sampling sites within La Union: Dulce Rocio and La Marsella.

Amphibian captive facility

At UVG's facilities, individuals from *P. matudai, B. conanti, B. nympha* and *B. mexicana*, were quarantined in plastic containers of 20.1cms by 15.7cms by 9.9cms (LxWxH) for 4 to 5 weeks. This was done following Amphibian Ark´s recommendations, animals were monitored carefully to check for any change in behaviour that could indicate that individuals were sick or infected with *B. dendrobatidis*. To maintain humidity levels, a moist non-bleached paper towel was put inside the container and changed every 48 hours. Animals were sprayed every day. Individuals were swabbed every week (Q1, Q2, Q3, Q4, Q5) during the quarantine stage using a rayon swab as described below (Figure 2). Samples were stored at -80°C until DNA extraction was done.

Individuals of *Plectrohyla matudai* from Finca La Marsella were randomly allocated on fully conditioned tanks of 45 cms x 45 cms x 60 cms (LxWxH). Five frogs (4 males, 1 female) were placed in tank A which had soil, rocks, sand and plants from the location where they were collected; 6 frogs (4 males, 2 females) were placed in tank B, which was prepared following the Amphibian Ark husbandry guideline protocols. Black soil, rocks sand and plants were commercially purchased and sterilize with bleach and high temperatures before preparing the tank. The set-up of both tanks was as similar as possible. A tank for *Bolitoglossa* salamanders (tank C) was prepared using clay pebbles for drainage, the soil, plants and rocks were sterilize as described before. The water source for all the tanks came from the same filter (iSpring CT10). Each one of these tanks (A, B and C) was one treatment.

The animals were fed once a week with crickets dusted with a vitamin and calcium suplement (Vetark Nutrobal). The cricket diet consisted of a mix of carrots, apples and cat pellets (Purina). Amphibians were misted twice a week. Daylight Blue Reptile Bulb 40W (Zoo Med DB-40) were used as UV source in a 12:12 photoperiod. The room temperature was kept between 18° to 20°C. The individuals were left in the tanks for 4 weeks before the first swab to let them get used to the new environment and to avoid excessive stress. Cutaneous swabs were taken every fortnight for tanks

A and B and every month for *Bolitoglossa* tanks (Figure 2). All the samples were stored at -80°C until processing.

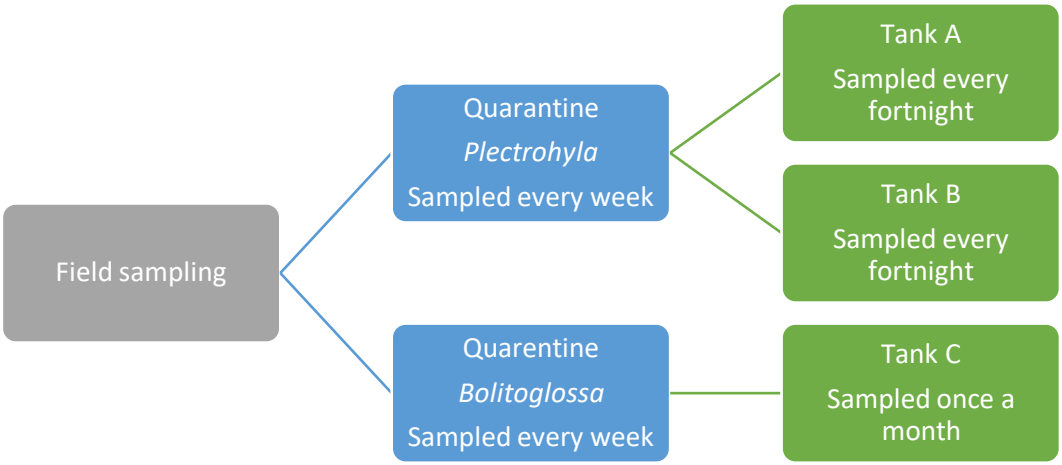


Figure 2. Diagram showing the different husbandry stages and the sampling frequency on each one.

DNA extraction and amplification

DNA extractions were done using the Qiagen DNeasy® PowerSoil® Kit, with the following modifications: C1 incubation was done at 65°C for 90 minutes. 50 ul of C6 were added to the filter and incubated for 10 minutes before elusion. Samples were quantified using a nanodrop™ (Thermo Fisher). For all the extraction sets a negative control was also extracted. Microbial community standard from ZymoBIOMICS ™ (D6300) was extracted as a positive control. DNA samples, extraction negatives and positive controls and PCR negatives were randomly assigned in triplicate into wells in 96 well plates. The plates also contained blanks which were wells left completely empty (Supp info figure 1).

PCR reactions were done to amplify the V4 region of the 16S gene using the primers 515F (Parada *et al.*, 2016) and 806R (Apprill *et al.*, 2015). Both primers were order with different tag combinations, added to the 5' -end of the primers. This allowed us to barcode each PCR product and to identify each sample after the sequencing run

(Supp info table 1). The primers were diluted to 100uM and used directly on the master mix. The PCR master mix was made to a final volume of 10ul; using 5ul of AmpliTaq Gold™ 360 Master Mix (applied biosystems), 2 ul of each primer (final concentration 1uM) and 1ul of DNA. The PCR program used was 95°C for 10 minutes, followed by 32 cycles of 95°C for 30 seconds, 50°C for 60 seconds and 72°C for 90 seconds, and a final extension of 72°C for 30 minutes. PCR fragments were visualized in 1% agarose gels. All the samples, controls and blanks from one plate were pooled into a 1.5ml Eppendorf tube and frozen at -80 until library preparation. Each one of these pools was treated as a single library.

Library preparation

Library preparation was done using Illumina® TruSeq® PCR-Free Library Prep kit. 1µg of each pooled sample was used as DNA input for 350bp insert size. Index adaptors 002, 004, 007 and 016 were used for the libraries. The quality of the final libraries was checked using a Qubit® dsDNA HS (High Sensitivity) Kit (Life technologies) and an Agilent 7500 Bioanalyzer chip with the High Sensitivity DNA Kit (Agilent Technologies). 4nM of each library was denatured using the MiSeq denature and dilute libraries protocol from Illumina® and pooled together. 10% PhiX DNA spike-in control was added. The library had a final concentration of 16pM. 600 ul were loaded into an Illumina® MiSeq Reagent Kit v3 (600 cycles) flow cell.

Chytridiomycosis testing

Samples were tested for chytrid fungus with real-time PCR (qPCR) to amplify the ITS-1- 5.8S rDNA region of *B. dendrobatidis*, using the primers ITS1-3 and 5.8S (Boyle *et al.*, 2004). PCR master mix consisted in 2.35 ul of dH2O, 10ul of SYBR® Green Master Mix (Sigma-Aldrich), 1ul of each primer, 0.05ul of probe Chytr MGB2 (Boyle *et al.*, 2004) 1.60ul of BSA (50mg/ml dH2O). Samples were run in duplicate. Bd DNA standards containing DNA from 100, 10, 1 and 0.1 genome equivalents (GE) and negative controls were run in triplicate. qPCR conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 60 cycles of 95°C for 15 secs and 60°C for 1 minute.

Data Analysis

Raw sequences were demultiplexed using the Cutadapt function (Martin, 2014) to obtained a set of demultiplexed fastq files. The R package DADA2 (Callahan *et al.*, 2016) was used to filter, dereplicate and to performed the quality controls of the sequences. The reads were trimmed at 220 and 180 base pair position for the forward and reverse, respectively. We filtered out the reads with more than 2 expected errors for the forward and 5 expected errors for the reverse sequences. Samples were merged, Amplicon Sequence Variant table (ASV) was constructed and chimeras were removed. To assign taxonomy the sequences were aligned against the 16S reference data base SILVA Small Subunit rRNA 138 (Quast *et al.*, 2013). A phyloseq object was created using the package Phyloseq (McMurdie & Holmes, 2013), including ecological information about each individual. Barplots were created to visualize the microbiome structure and relative abundance of the samples during the different stages of the study.

Alpha diversity was calculated using Shannon and Simpson indices on Phyloseq. To compare the diversity among the species, sites (from wild specimens) and sampling stages (Field, Quarantine, Tanks) Kruskal-Wallis tests or a Wilcoxon tests were performed. Beta diversity analysis were obtained using weighted and unweighted UniFrac distances. The weighted unifrac considers the relative abundance and the unweighted only takes into consideration presence/absence of the taxa. The of the beta diversity indices were visualized using a non-metric multidimensional scaling (NMDS). We performed an analysis of similarity (ANOSIM) and a permutational multivariate analysis of variance (PERMANOVA) using VEGAN (Dixon, 2003) to determine if there was any significant difference among beta diversity and the sampling stages. Additionally, a PERMANOVA was done to determine the effects of the tank design on the bacterial diversity.

B. dendrobatidis analysis

Infection intensity was defined as the number of zoospore equivalents ZE (Vredenburg *et al.*, 2010). These were calculated by multiplying by 20 the quantity values obtained in the output file from the qPCR (Bletz *et al.*, 2015) to remove the dilution factor from the extractions. A sample was considered positive for Bd when the ZE was ≥1 and negative when the ZE was <1.

Results

Microbiome from the wild

In the wild we obtained amphibian skin samples from 37 individuals from 7 species. 3 *Bolitoglossa conanti*, 2 *B. dofleini*, 1 *B. mexicana*, 5 *B. nympha*, 2 *Bromeliohyla*, 2 *Ecnomiohyla* and 22 *P. matudai*. Skin-bacterial composition from wild individuals was dominated by bacteria from the Phylum Proteobacteria, followed by Bacteriota and Actinobacteriota Phyla (Figure 1, supplementary information). When analysing the data at the order level we found that Pseudomonales and Burkholderiales were the most prominent orders across all the species (Figure 3). *B. dofleini* was dominated mainly by Burkholderiales, while in the other 3 species of *Bolitolgossa,* Pseudomonales and Rickettsiales were also abundant. *Ecnomiohyla* tree frogs had a significant abundance of the order Bacteroidales, which was present in very low abundance in the other species. In *Plectrohyla* frogs the order Micrococcales was very prominent.

When analysing the bacterial composition at the family level salamanders showed high abundance of Comamonadaceae and Dermabacteraceae. *B. mexicana* also showed high prominence of Moraxellaceae family. Frogs from the genera *Bromeliohyla* and *Plectrohyla* tree frogs seem to have a very homogeneous composition at the family level. While *Ecnomiohyla* samples were dominated by the families Enterobacteriaceae and Comamonadaceae (Figure 2, supplementary information).

At the genera level *Xylophilus* was the most abundant genus for *B. dofleini*, while in *B. mexicana, B. conanti* and *B. nympha* genera *Acinetobacter* and *Pseudomonas* were the most abundant. In the case of *Bromeliohyla* and *Ecnomiohyla* frogs, genera *Pseudomonas* and *Escherichia/Shigella* were the most prominent. *P. matudai* was dominated by the genera *Acinetobacter, Chryseobacterium* and *Pseudomonas* (Figure 4), 3 important genera that can have anti-Bd properties (Woodhams *et al.,* 2015).

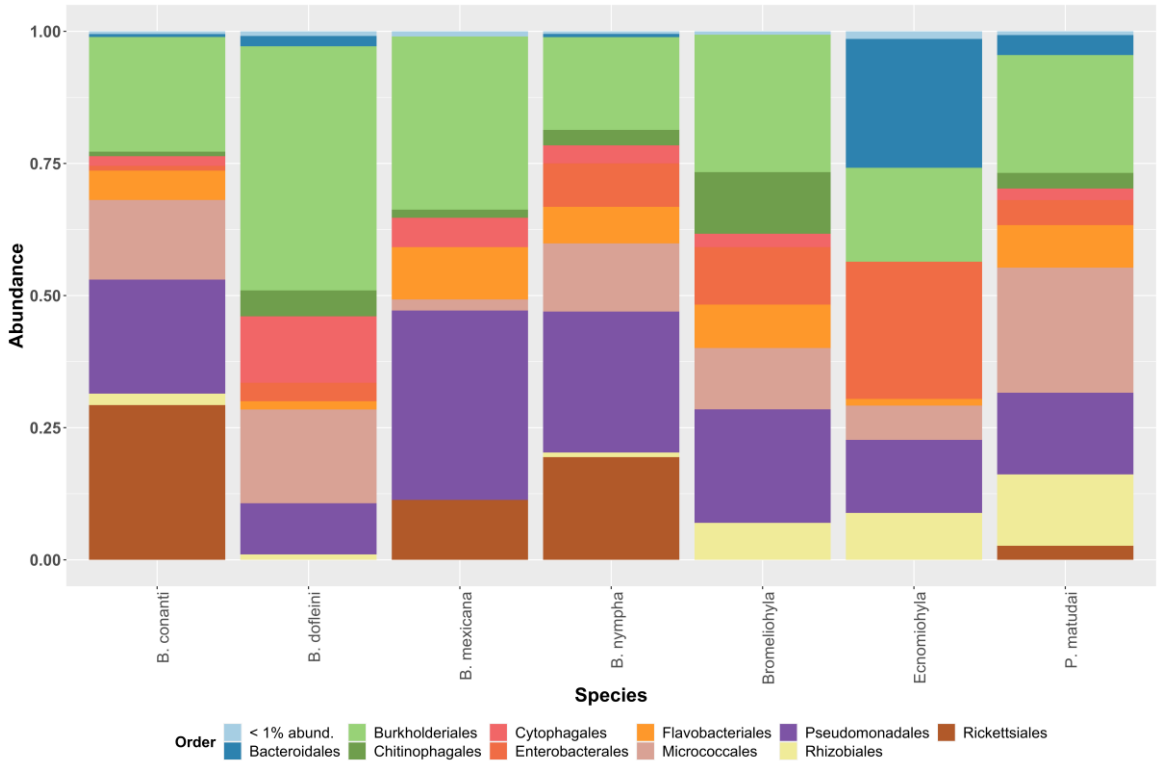


Figure 3. Top 10 orders present in samples of wild amphibians from La Unión, Zacapa. *B. conanti, B. dofleini, B. mexicana, B. nympha, Bromeliohyla* spp, *Echnomiohyla* and *P. matudai.*

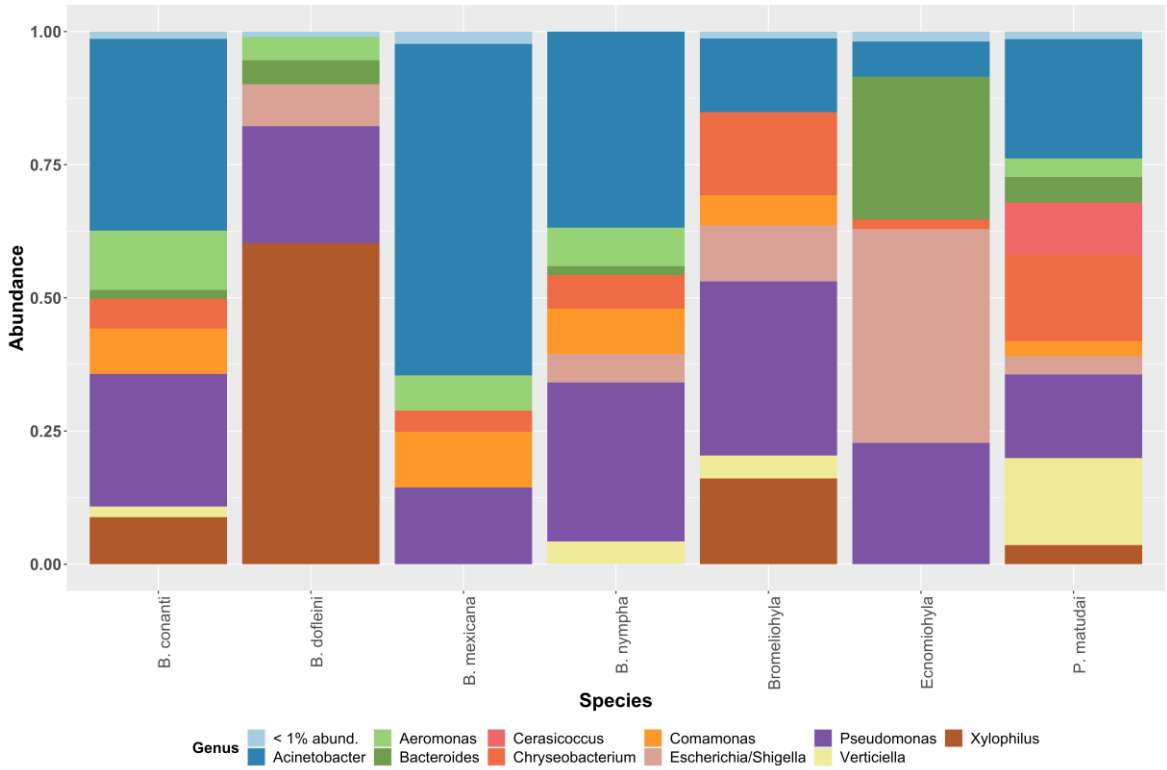


Figure 4. Top 10 genera present in samples of wild amphibians from La Unión, Zacapa. *B. conanti, B. dofleini, B. mexicana, B. nympha, Bromeliohyla* spp, *Echnomiohyla* and *P. matudai*

Overall *B. dofleini* and *Echnomiohyla* presented the highest levels of alpha diversity in the wild (Figure 5). We could not find any significant different between the alpha diversity in the different species (Shannon: KW = 5.70, df = 6, p-value = 0.46, Simpson: KW = 6.1, df = 6, p-value = 0.41). Beta diversity was measured using weighted UniFrac distance, there was no significative differences among species (PseudoF = 0.67, R^2= 0.12, p-value = 0.89) or the different genera sampled in the wild (PseudoF = 0.52, R^2= 0.05, p-value = 0.84).

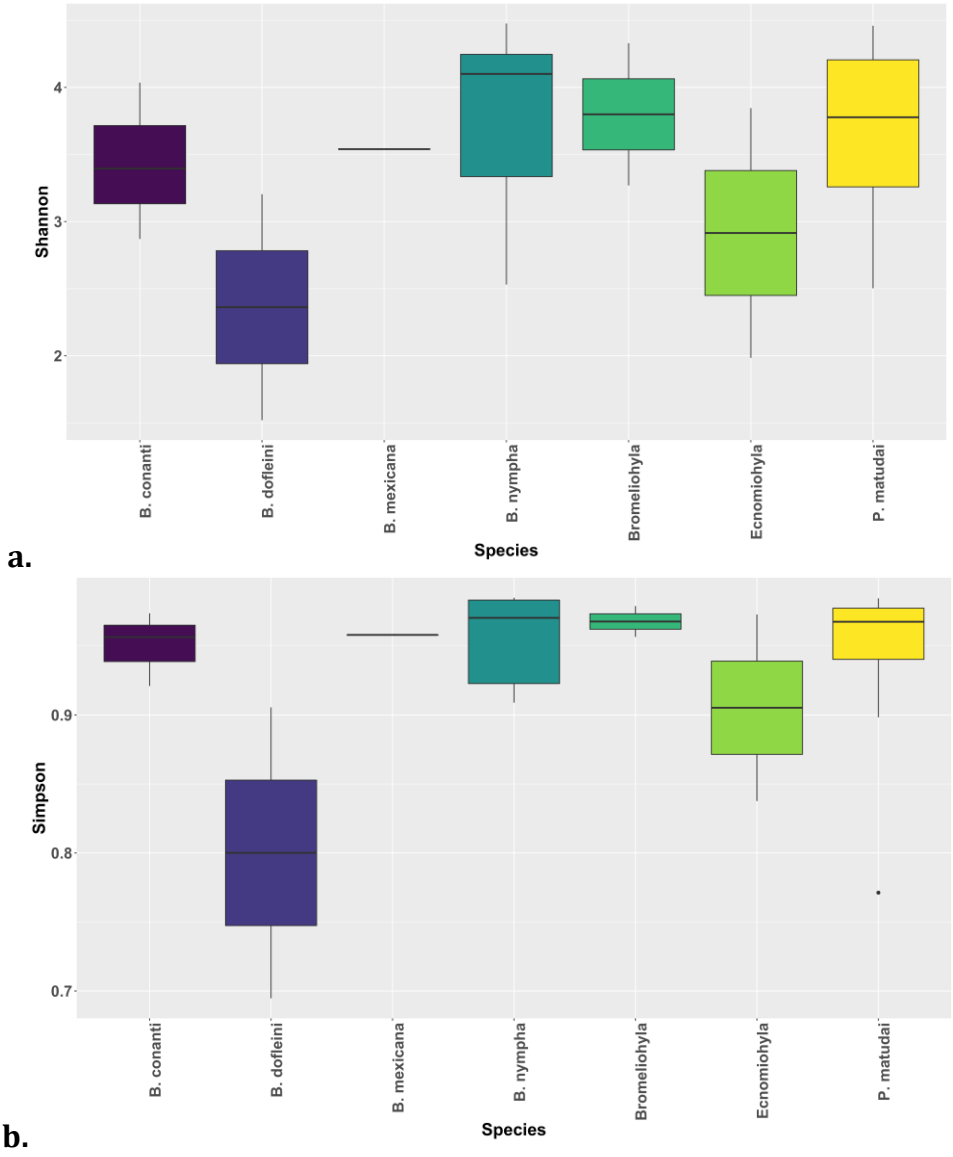


Figure 5. Alpha diversity for wild amphibians. a. Shannon and b. Simpson indices. Line in boxes represent the median, whiskers (vertical lines) represent the maximum and minimum values, boxes go from the first quartile to the third quartile.

Bolitoglossa microbiome

We sampled 4 different species of *Bolitoglossa* in the field but only 3 were kept during the whole study, since keeping *B. dofleini* in captivity can be very challenging. There was no significative difference between the alpha diversity and the different sampling stages (field, quarantine, tanks) of *Bolitoglossa* salamanders (Shannon: K-W chi-squared = 12.517, df = 8, p-value = 0.1296; Simpson:K-W chi-squared = 15.255, df = 8, p-value = 0.05437). Simpson index shows that during quarantine diversity levels droped. When animals were transferred into the experimental tank (tank C) diversity levels went back to similar levels as when they were collected in the wild.

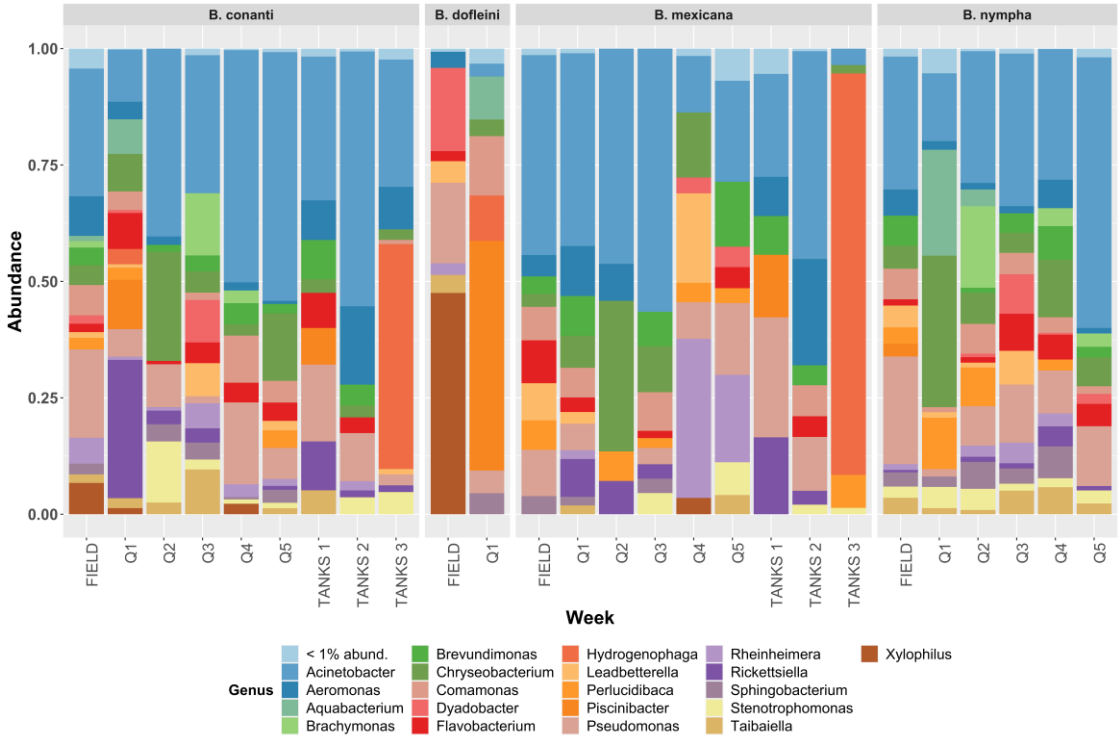


Figure 6. Top 20 Genera identified for *Bolitoglossa* spp. individuals across the study. Animals sampled every week for 5 weeks during quarantine. **Labels:** Q1 – Q5 (sample taken every week during quarantine); during experimental tank stage animals were sampled every month for 3 months, TANKS1, TANKS, 2 and TANKS 3.

In the case of beta diversity, we did find significantly differences between the different sampling stages for both weighted (PseudoF=1.74, R^2=0.22, p-value= 0.024) and unweighted UniFrac distances (PseudoF=1.32, R^2=0.17, p-value= 0.008). Showing that captivity can impact both the presence and abundance of the different

bacterial groups. The ANOSIM results also showed a significant difference among the different stages of the study and the community composition (R=0.33, p-value=0.001).

Plectrohyla microbiome

For *P. matudai* we only analyse the data from 16 frogs that were captured on the same river at Finca La Marsella. We found that genera *Acinetobacter, Chryseobacterium* and *Peseudomonas* were the most common in the wild samples. During quarantine stage, bacterial communities became more homogenous and *Chryseobacterium* and *Peseudomonas* became less abundant. Over time the genera *Rickettsiella* and *Aeromonas*, whose abundance was very low in the field became more prominent (Figure 7).

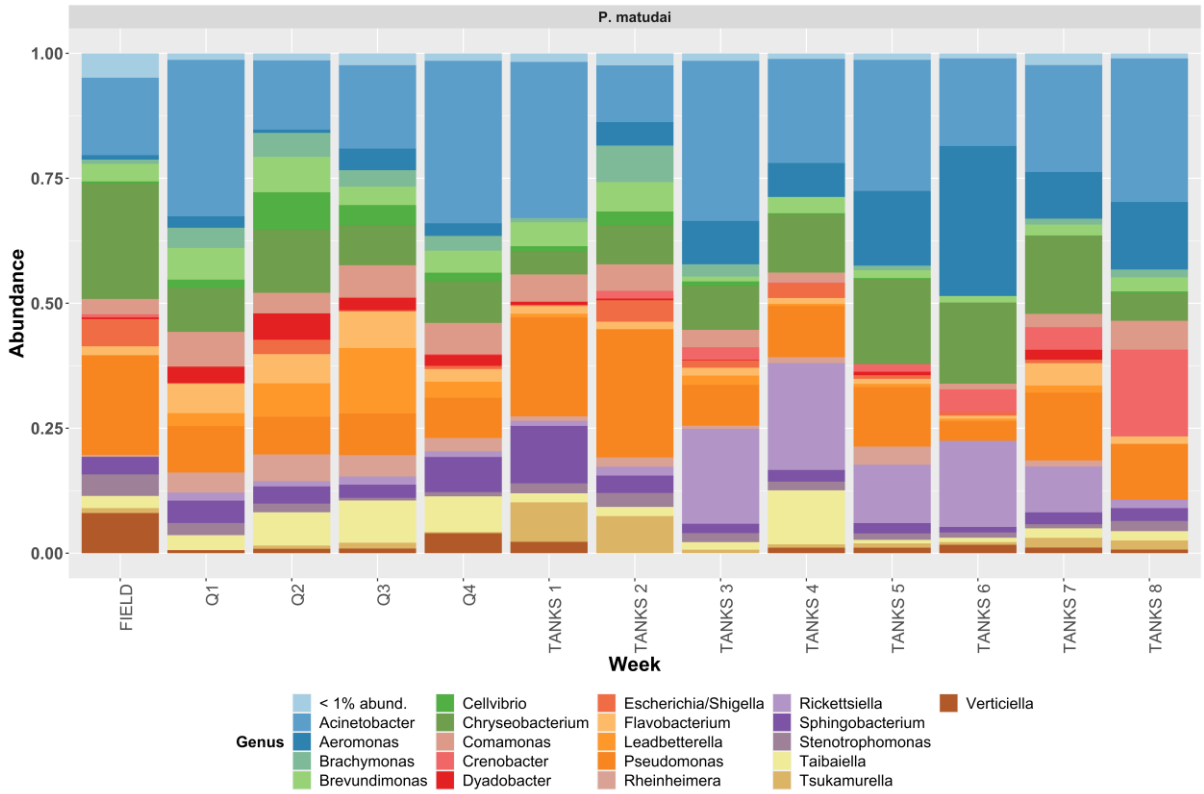


Figure 7. Top 20 Genus identified for *Plectrohyla matudai* individuals during the different stages of the study. **Labels:** Q1 - Q4 (samples taken every week during quarantine); TANKS 1- TANKS 8 (samples taken every fortnight while animals were in tanks); A-B (identification code for the 2 different tanks).

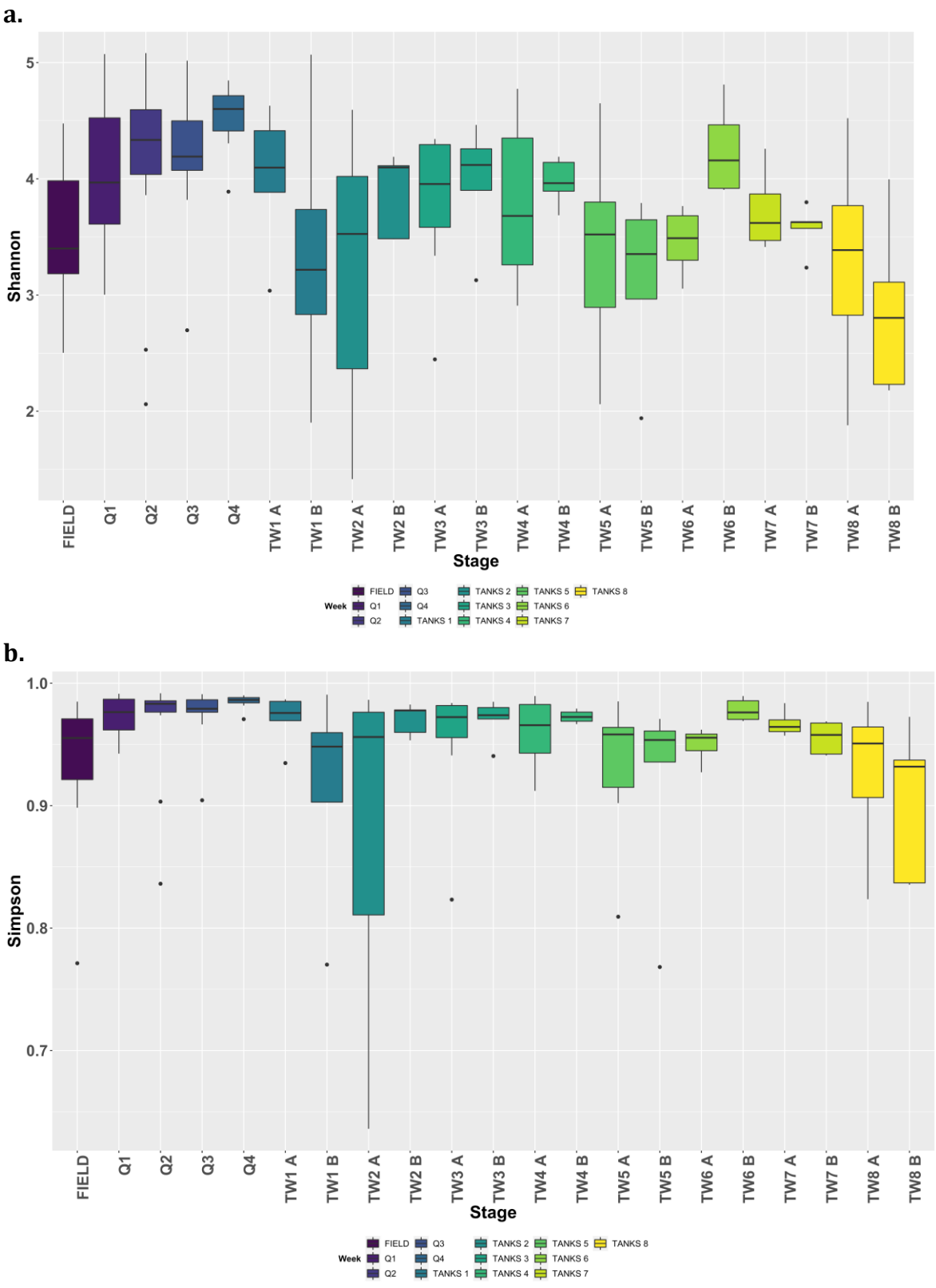
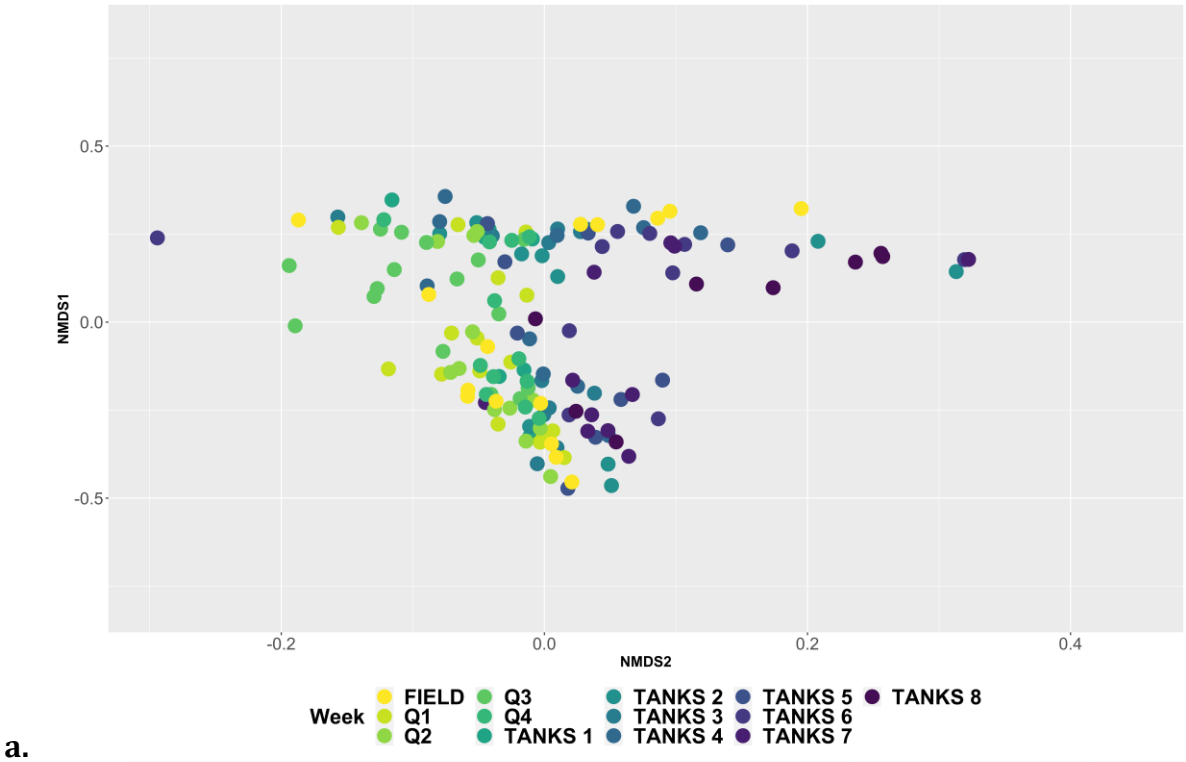


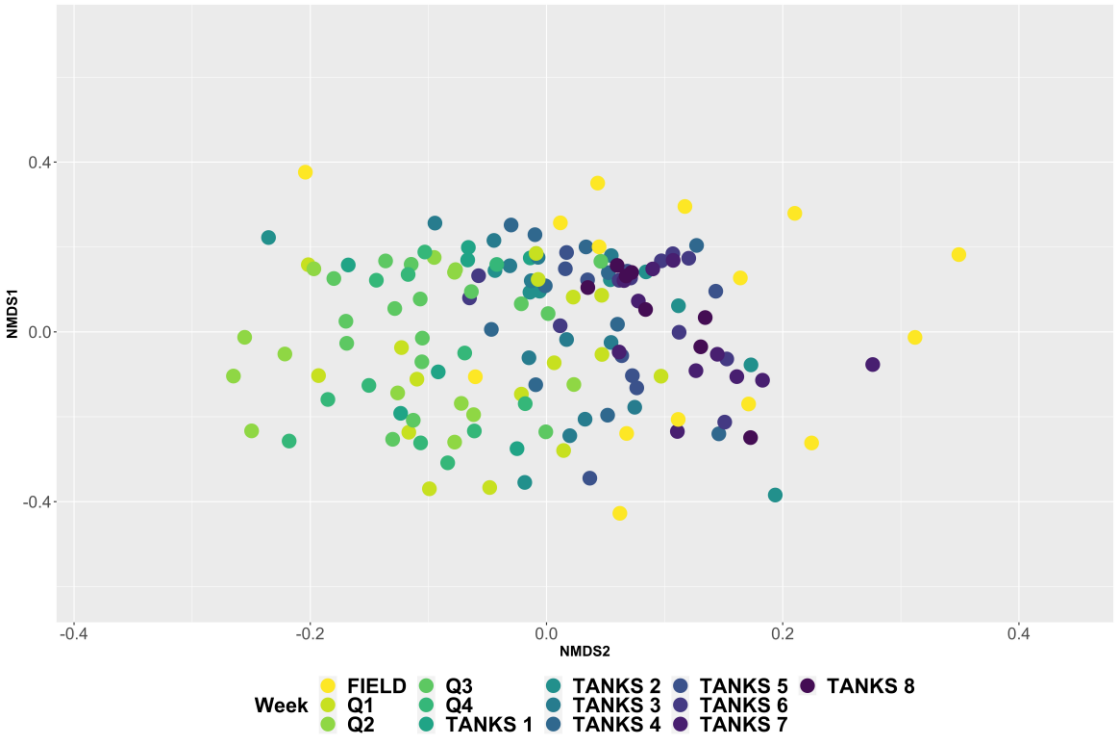
Figure 8. Alpha diversity indices a. Shannon and b. Simpson for *P. matudai* samples. Line in boxes represent the median, whiskers (vertical lines) represent the maximum and minimum values, boxes go from the first quartile to the third quartile. **Labels:** Q1 - Q4 (samples taken every week during quarantine); TW1- TW8 (samples taken every fortnight while animals were in tanks); A-B (identification code for the 2 different tanks).

We found significant differences between the alpha diversity of *Plectrohyla* individuals during the different sampling stages (Shannon K-W chi-squared=50.5, df=14, p-value<0.001; Simpson K-W chi-squared=45.6, df=14, p-value<0.001). After performing the Dunn's post hoc test (Table 1, Supplementary information), we identify that the significant differences were mainly between the field and quarantine stages and between experimental tanks and quarantine stages. When testing the two different tanks where the frogs were housed, we did not find a significant difference between them and the alpha diversity (Shannon: W = 12, p-value = 0.99; Simpson: W = 12, p-value = 0.97).

Beta diversity was also significant when testing for both weighted (Pseudo = 2.19, R^2=0.15, p-value < 0.001) and unweighted distances (Pseudo = 1.89, R^2=0.13, p-value < 0.001) during the husbandry stages. NMDS for both distances showed that field samples are more scattered around the plot, while the data points for the quarantine and experimental tanks cluster closer within their category, especially for weighted distances (Figure 9). We could not find any significant results when performing PERMANOVAS to compare the bacterial communities of the two different experimental tanks (A substrate and furnishings from site; B sterile substrate and furnishings) (Weighted: Pseudo = 0.38, R^2=0.004, p-value =0.81; Unweighted: Pseudo = 0.60, R^2=0.007, p-value =0.99).



a.



b.

Figure 9. Beta diversity for *P. matudai* along different sampling stages using the a. weighted and b. unweighted UniFrac distances. **Labels:** Q1 - Q4 (samples taken every week during quarantine); TANKS 1- 8 (samples taken every fortnight while animals were in tanks).

Only 1 *B. conanti*, 2 *Plectrohyla matudai* and 2 *Echnomiohyla* tested positive for Bd in the field. We kept all the animals in quarantine and checked for signs of infection, *B. conanti* (B9) showed signs of Bd infection (skin ulcers, dropped tail) and died within a couple of weeks. When analysing the samples obtained during quarantine for B9 (*B. conanti*) and P8 (*P. matudai*) we found that infection increased during week 1 and 2 in quarantine then it deceased during week 3 and finally it increased again. The salamander died during quarantine. The frog survived the quarantine phase and was transferred to a tank, *P. matudai* has shown to be resistant to Bd (Cheng *et al.,* 2011). During quarantine, 4 frogs died we did not detect Bd infection on these individuals, so we associate these deaths to stress due to captivity.

Discussion

We collected amphibians from the forests of La Unión, Zacapa to characterize the skin microbiome and to determine how it changed when the animals were taken into captivity. La Unión is a municipal forest and one of the last remains of cloud forest in Eastern Guatemala, habitat of several species of amphibians. Unfortunately, this forest is under the constant threat of deforestation through illegal logging, putting its future in doubt.

We could not find any significant difference between the alpha diversity and the different sampling stages for *Bolitoglossa* salamanders. We did find significant differences when testing beta diversity during the different sampling phases. Showing that the composition and structure of the bacterial communities did get affected by captive conditions, especially quarantine. Bd could not be detected in all the wild samples taken from *Bolitoglossa* salamanders, however during quarantine some individuals started showing signs of infection, like ulcers, lack of appetite and they dropped the tail before dying. Bd infection in these animals was very low in the field, however stress or a change in the microbiome could have contribute to increase the infection levels. It has been documented that stress and handling an amphibian can have an impact on the bacterial and fungal communities (Antwis *et al.*, 2014b) and if the microbiome is disrupted it could give Bd an advantage to colonize the host (Loudon *et al.*, 2014).

Alpha diversity of samples taken from *P. matudai* were significantly different along the sampling stages. We determined that the differences in the community richness were mainly between the field and quarantine, and quarantine and tanks stages. We could not detect any significant difference between samples taken in the wild and the ones taken when animals were in tanks. Beta diversity was also significantly different along the sampling stages, showing that the different husbandry phases can have a significant effect on the composition and structure of the bacterial communities.

Quarantine can be very stressful for animals since they are housed within a plastic box with only a wet paper towel and not much space to hide. We had to change the paper towels every 48 hours or less, which sometimes involved holding the animal to clean the box. This could have added extra stress to the animal, and as discussed above this can have an impact on the microbiome and thus the health of the individuals (Antwis *et al.*, 2014b). Bates *et al.* (2019) recorded changes in the diversity of bacterial and fungal communities after the animals were in captivity for only 2 weeks.

During the tank stages diversity levels seemed to go back to levels similar as the ones observed in the wild. When testing alpha diversity and beta diversity between the two different tanks housing *P. matudai* we could not find any differences. This could mean that *P. matudai* microbiome might be related to the host more than the environment, as showed in other tropical species (Mckenzie *et al.* 2012; Kueneman *et al.*, 2014; Abarca, *et al.*, 2018; Varela *et al.*, 2018). The retention of key groups that have anti-Bd properties could potentially explain why the frog is Bd resistant and show low levels of infection in laboratory conditions (Cheng *et al.*, 2011).

Like in other microbiome studies with amphibians, Proteobacteria was the most prominent phylum in all the species. Among bacteria genera that we identified was *Pseudomonas, Acinetobacter, Aeromonas,* and *Chryseobacterium,* which can have anti-Bd properties (Woodhams *et al.*, 2015), potentially protecting the animals from the infection. Previous studies have detected Bd in several species of La Unión, however infection levels across the different species was low (Ruano *et al.* 2011). We only detected Bd in a few individuals and as in previous studies levels of infection were low. However, we did find considerably less salamanders than 9-10 years ago (personal obs), which opens the question if a new outbreak could potentially be affecting the populations of salamanders.

Rickettsiella genus, that was present in very low abundances in wild wild *P. matudai*, became more prominent when the animals were in the tanks. This bacteria could have been in the crickets as it is known they are pathogens of arthropods (Louis *et al.*, 1986; Cordaux *et al.*, 2007; Wang & Chandler, 2016) and can survive in the soil for a long time (Weiss *et al.*, 1984).

Amphibians are endangered and more *ex-situ* programs will need to be implemented in the future; it is important to understand the different stressors that the individuals might face while in captivity and ways to reduce it as much as possible. We proved that a simpler environment like a wet paper towel can stress the animal and can reduce the diversity of the skin microbiome. It is important to understand what the health consequences for the individuals are. It is known that shifts in the bacterial communities can give an advantage for pathogens like Bd (Jani & Briggs, 2014) and that less diverse bacterial communities can be related to higher infections of Bd and Bsal (López *et al.*, 2017; Ellison *et al.*, 2019). Highlighting the importance of monitoring changes in the microbiome, especially if the aim of the project is the reintroduction of the amphibians into the wild.

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Supplementary information

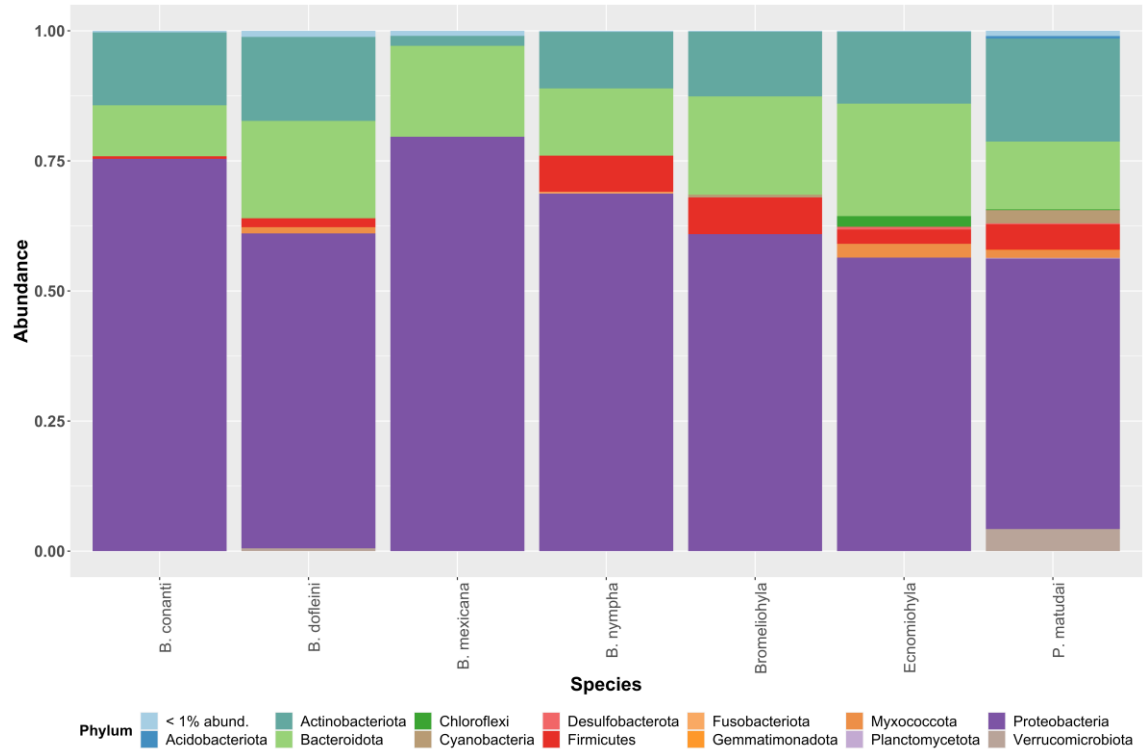

Figure 1. Phyla identified for the different species collected at La Union forest

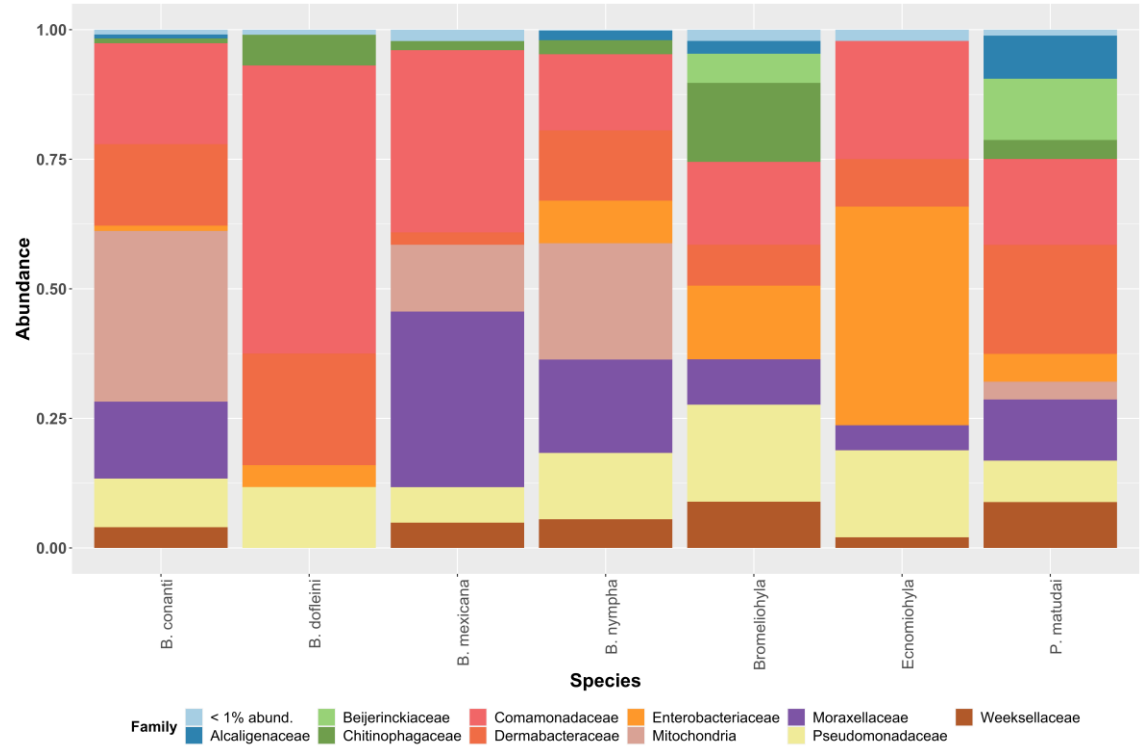

Figure 2. Top 10 families present in wild amphibians at La Union, Zacapa

Table 1. Dunn's post hoc test results for Shannon index along the different husbandry stages. Highlighted values were significant.

chi2	Z	P	P.adjusted	comparison
45.6524531	-2.1367189	0.01631043	0.01631043	FIELD - Q1
45.6524531	-2.8513185	0.00217692	0.00217692	FIELD - Q2
45.6524531	-0.7493454	0.22682453	0.22682453	Q1 - Q2
45.6524531	-2.8075054	0.00249634	0.00249634	FIELD - Q3
45.6524531	-0.6707865	0.25117827	0.25117827	Q1 - Q3
45.6524531	0.0894667	0.46435551	0.46435551	Q2 - Q3
45.6524531	-4.1878662	1.41E-05	1.41E-05	FIELD - Q4
45.6524531	-2.2096489	0.01356477	0.01356477	Q1 - Q4
45.6524531	-1.4833801	0.06898672	0.06898672	Q2 - Q4
45.6524531	-1.5886213	0.05607296	0.05607296	Q3 - Q4
45.6524531	-0.9177248	0.17938148	0.17938148	FIELD - T1
45.6524531	0.89534127	0.18530234	0.18530234	Q1 - T1
45.6524531	1.52351857	0.06381451	0.06381451	Q2 - T1
45.6524531	1.46452251	0.07152562	0.07152562	Q3 - T1
45.6524531	2.75963008	0.00289334	0.00289334	Q4 - T1
45.6524531	-0.5408675	0.29429945	0.29429945	FIELD - T2
45.6524531	1.38788648	0.08258581	0.08258581	Q1 - T2
45.6524531	2.04785584	0.02028706	0.02028706	Q2 - T2
45.6524531	1.99338598	0.0231096	0.0231096	Q3 - T2
45.6524531	3.32377907	0.00044403	0.00044403	Q4 - T2
45.6524531	0.3794302	0.35218421	0.35218421	T1 - T2
45.6524531	-1.4013826	0.08054985	0.08054985	FIELD - T3
45.6524531	0.57683466	0.28202557	0.28202557	Q1 - T3
45.6524531	1.26412975	0.10309173	0.10309173	Q2 - T3
45.6524531	1.19786231	0.11548531	0.11548531	Q3 - T3
45.6524531	2.60651672	0.00457342	0.00457342	Q4 - T3
45.6524531	-0.3464645	0.36449683	0.36449683	T1 - T3
45.6524531	-0.7745554	0.2193012	0.2193012	T2 - T3
45.6524531	-1.2187301	0.11147333	0.11147333	FIELD - T4
45.6524531	0.71002392	0.23884465	0.23884465	Q1 - T4
45.6524531	1.37901545	0.083945	0.083945	Q2 - T4
45.6524531	1.31552342	0.09416703	0.09416703	Q3 - T4
45.6524531	2.68773056	0.00359697	0.00359697	Q4 - T4
45.6524531	-0.2112736	0.41633688	0.41633688	T1 - T4
45.6524531	-0.6226565	0.26675513	0.26675513	T2 - T4
45.6524531	0.13850692	0.4449199	0.4449199	T3 - T4
45.6524531	0.58725411	0.27851653	0.27851653	FIELD - T5
45.6524531	2.51600811	0.00593462	0.00593462	Q1 - T5

45.6524531	3.16096248	0.00078624	0.00078624	Q2 - T5
45.6524531	3.12150761	0.00089964	0.00089964	Q3 - T5
45.6524531	4.38231237	5.87E-06	5.87E-06	Q4 - T5
45.6524531	1.36249937	0.08652017	0.08652017	T1 - T5
45.6524531	1.03624589	0.15004372	0.15004372	T2 - T5
45.6524531	1.83308873	0.03339469	0.03339469	T3 - T5
45.6524531	1.65890241	0.04856773	0.04856773	T4 - T5
45.6524531	-0.9169081	0.17959541	0.17959541	FIELD - T6
45.6524531	1.01184594	0.15580586	0.15580586	Q1 - T6
45.6524531	1.6768203	0.04678881	0.04678881	Q2 - T6
45.6524531	1.61734543	0.05290187	0.05290187	Q3 - T6
45.6524531	2.97093464	0.00148447	0.00148447	Q4 - T6
45.6524531	0.05174048	0.47936774	0.47936774	T1 - T6
45.6524531	-0.3454153	0.36489109	0.36489109	T2 - T6
45.6524531	0.421711	0.33661799	0.33661799	T3 - T6
45.6524531	0.27724122	0.39079744	0.39079744	T4 - T6
45.6524531	-1.3816612	0.08353788	0.08353788	T5 - T6
45.6524531	-0.0708168	0.47177176	0.47177176	FIELD - T7
45.6524531	1.85793716	0.03158897	0.03158897	Q1 - T7
45.6524531	2.51165028	0.00600841	0.00600841	Q2 - T7
45.6524531	2.46343666	0.00688061	0.00688061	Q3 - T7
45.6524531	3.76483461	8.33E-05	8.33E-05	Q4 - T7
45.6524531	0.78904236	0.21504363	0.21504363	T1 - T7
45.6524531	0.43176912	0.33295461	0.33295461	T2 - T7
45.6524531	1.21561097	0.11206658	0.11206658	T3 - T7
45.6524531	1.05442564	0.14584404	0.14584404	T4 - T7
45.6524531	-0.6044768	0.27276336	0.27276336	T5 - T7
45.6524531	0.77718442	0.21852499	0.21852499	T6 - T7
45.6524531	0.97813836	0.16400295	0.16400295	FIELD - T8
45.6524531	2.90689236	0.00182519	0.00182519	Q1 - T8
45.6524531	3.54664417	0.00019509	0.00019509	Q2 - T8
45.6524531	3.51239186	0.00022205	0.00022205	Q3 - T8
45.6524531	4.74908487	1.02E-06	1.02E-06	Q4 - T8
45.6524531	1.70312421	0.04427241	0.04427241	T1 - T8
45.6524531	1.395296	0.0814633	0.0814633	T2 - T8
45.6524531	2.19986123	0.01390837	0.01390837	T3 - T8
45.6524531	2.01795252	0.0217981	0.0217981	T4 - T8
45.6524531	0.35905011	0.3597788	0.3597788	T5 - T8
45.6524531	1.74071129	0.0408671	0.0408671	T6 - T8
45.6524531	0.96352688	0.16764159	0.16764159	T7 - T8

Table 2. Dunn's post hoc test results for Simpson index along the different husbandry stages. Highlighted values were significant.

chi2	Z	P	P.adjusted	comparison
50.060972	-2.8866431	0.00194688	0.00194688	FIELD - Q1
50.060972	-3.3016178	0.00048064	0.00048064	FIELD - Q2
50.060972	-0.4619151	0.32207111	0.32207111	Q1 - Q2
50.060972	-3.4029226	0.00033335	0.00033335	FIELD - Q3
50.060972	-0.5162795	0.3028296	0.3028296	Q1 - Q3
50.060972	-0.0459691	0.48166745	0.48166745	Q2 - Q3
50.060972	-4.5344322	2.89E-06	2.89E-06	FIELD - Q4
50.060972	-1.86192	0.03130719	0.03130719	Q1 - Q4
50.060972	-1.4072388	0.0796783	0.0796783	Q2 - Q4
50.060972	-1.383938	0.08318872	0.08318872	Q3 - Q4
50.060972	-1.333419	0.09119718	0.09119718	FIELD - T1
50.060972	1.11597894	0.13221557	0.13221557	Q1 - T1
50.060972	1.49655364	0.06725472	0.06725472	Q2 - T1
50.060972	1.55405663	0.06008545	0.06008545	Q3 - T1
50.060972	2.66697096	0.00382691	0.00382691	Q4 - T1
50.060972	-0.9583467	0.16894397	0.16894397	FIELD - T2
50.060972	1.64734209	0.04974388	0.04974388	Q1 - T2
50.060972	2.04362473	0.02049532	0.02049532	Q2 - T2
50.060972	2.1133726	0.01728444	0.01728444	Q3 - T2
50.060972	3.24910914	0.00057884	0.00057884	Q4 - T2
50.060972	0.40098874	0.34421421	0.34421421	T1 - T2
50.060972	-1.7246891	0.04229176	0.04229176	FIELD - T3
50.060972	0.94782309	0.17160976	0.17160976	Q1 - T3
50.060972	1.36320523	0.08640892	0.08640892	Q2 - T3
50.060972	1.42580504	0.07696231	0.07696231	Q3 - T3
50.060972	2.62827395	0.00429097	0.00429097	Q4 - T3
50.060972	-0.2336621	0.40762365	0.40762365	T1 - T3
50.060972	-0.6786065	0.24869361	0.24869361	T2 - T3
50.060972	-1.6708446	0.04737619	0.04737619	FIELD - T4
50.060972	0.93484421	0.17493429	0.17493429	Q1 - T4
50.060972	1.34061001	0.09002355	0.09002355	Q2 - T4
50.060972	1.40087472	0.08062577	0.08062577	Q3 - T4
50.060972	2.5805618	0.00493198	0.00493198	Q4 - T4
50.060972	-0.2198971	0.41297567	0.41297567	T1 - T4
50.060972	-0.6544711	0.25640417	0.25640417	T2 - T4
50.060972	0.01005916	0.49598704	0.49598704	T3 - T4
50.060972	0.23409761	0.4074546	0.4074546	FIELD - T5
50.060972	2.83978645	0.00225719	0.00225719	Q1 - T5

50.060972	3.22019798	0.00064051	0.00064051	Q2 - T5
50.060972	3.30581696	0.0004735	0.0004735	Q3 - T5
50.060972	4.36799741	6.27E-06	6.27E-06	Q4 - T5
50.060972	1.44011009	0.07491813	0.07491813	T1 - T5
50.060972	1.09533008	0.13668603	0.13668603	T2 - T5
50.060972	1.79749477	0.03612855	0.03612855	T3 - T5
50.060972	1.74980117	0.04007631	0.04007631	T4 - T5
50.060972	-1.0820443	0.13961642	0.13961642	FIELD - T6
50.060972	1.52364454	0.06379877	0.06379877	Q1 - T6
50.060972	1.92157357	0.02732972	0.02732972	Q2 - T6
50.060972	1.98967505	0.02331337	0.02331337	Q3 - T6
50.060972	3.1330419	0.00086502	0.00086502	Q4 - T6
50.060972	0.29319607	0.38468614	0.38468614	T1 - T6
50.060972	-0.1136235	0.45476815	0.45476815	T2 - T6
50.060972	0.56253926	0.28687433	0.28687433	T3 - T6
50.060972	0.54084763	0.2943063	0.2943063	T4 - T6
50.060972	-1.2089535	0.11334035	0.11334035	T5 - T6
50.060972	-0.592202	0.27685766	0.27685766	FIELD - T7
50.060972	2.01348683	0.02203172	0.02203172	Q1 - T7
50.060972	2.40489619	0.00808853	0.00808853	Q2 - T7
50.060972	2.47951734	0.00657802	0.00657802	Q3 - T7
50.060972	3.59266819	0.00016365	0.00016365	Q4 - T7
50.060972	0.72005505	0.23574555	0.23574555	T1 - T7
50.060972	0.33632542	0.36831274	0.36831274	T2 - T7
50.060972	1.02216556	0.15335127	0.15335127	T3 - T7
50.060972	0.99079651	0.16089248	0.16089248	T4 - T7
50.060972	-0.7590047	0.22392488	0.22392488	T5 - T7
50.060972	0.44994887	0.32637365	0.32637365	T6 - T7
50.060972	0.58045074	0.28080535	0.28080535	FIELD - T8
50.060972	3.18613958	0.00072093	0.00072093	Q1 - T8
50.060972	3.56194125	0.00018406	0.00018406	Q2 - T8
50.060972	3.65217009	0.00013002	0.00013002	Q3 - T8
50.060972	4.6929857	1.35E-06	1.35E-06	Q4 - T8
50.060972	1.74192958	0.04076038	0.04076038	T1 - T8
50.060972	1.41347575	0.07875794	0.07875794	T2 - T8
50.060972	2.12248306	0.0168986	0.0168986	T3 - T8
50.060972	2.06794684	0.01932251	0.01932251	T4 - T8
50.060972	0.31814567	0.37518722	0.37518722	T5 - T8
50.060972	1.5270992	0.06336817	0.06336817	T6 - T8
50.060972	1.07715033	0.14070656	0.14070656	T7 - T8

Chapter 6 - General Discussion

Amphibians are the most threated vertebrates on the planet, at least 41% of the described species are endangered and several have become extinct (IUCN 2020). Amphibian populations in Mesoamerica are threatened by various pressures, from habitat destruction and pollution to infectious diseases such as *B. dendrobatidis* (Berger *et al.*, 1998; Stuart *et al.*, 2004; Lips *et al.*, 2005; Mendelson *et al.*, 2006). Amphibians play an important role in different ecosystems and it is crucial to understand more about the impacts that these pressures can have on the diversity of the different species before is too late. I studied the population genetics and described the skin-bacterial communities of Mesoamerican species to determine its genetic diversity and identified some of the pressures threatening this group in the region.

Developing genetic tools for conservation is becoming more important to answer ecological and conservation questions. In chapter 2, I developed microsatellite markers for *Agalychnis moreletii* to study the populations genetics and connectivity across its distribution range. The markers developed for this project could potentially be used to study other *Agalychnis* species in the Neotropics and help to determine their genetic diversity and population structure, especially for critically endangered species. Most of the visited sites were threatened, and even though the numbers of individuals of *A. moreletii* in some were high, the genetic diversity levels were low, and several populations showed evidence of inbreeding and bottleneck events. I also found a high degree of population structure and along the distribution range as well as no evidence for gene flow between several populations. The isolation of the populations is mainly due to habitat fragmentation caused by roads, urbanization and plantations of sugar cane, coffee and palm oil. Having large population numbers does not always means the genetic diversity is high (Kotzé *et al.*, 2019).

Due to the high phenotypic and morphological diversity of *Agalychnis* frogs there is still debate about the taxonomic status of the different species (Duellman 2001). For example, it was determined that *A. callidryas* is a complex of five species along its distribution in Central America (Solano-Flórez 2012). In chapter 2 and 3, I concluded that *A. moreletii* populations should be separated into different Evolutionary Significant Units (ESUs) for conservation management. Using microsatellite markers, it was determined that *A. moreletii* in Mexico and Central America is comprised of three ESUs. However, using mitochondrial markers we detected that Atlantic populations should be further split into two units, separating the Eastern Chiapas populations (LBEL, POM and TyL) from the rest of ATL. Concluding that *A. moreletii* populations should be separated into four ESUs, Atlantic populations (ATL), Pacific populations (PAC), Eastern Chiapas populations (CHI) and Veracruz populations (VER). The different ESUs are separated by geographical barriers, nonetheless we could not detect any signal of isolation by distance (IBD) using microsatellites which clearly shows that it is a species complex and should be managed accordingly. Each of these ESUs should have different management plans since the habitat is very different in all of them, from extremely fragmented habitat in populations associated with the Pacific coast and Veracruz in Mexico, to pristine rain forests in Belize.

Despite a decreasing population trend IUCN classifies *A. moreletii* as Least Concern (IUCN 2017). I found that genetic diversity is very low and what was thought of as one species with a large geographical range is actually a complex of four, some of which are highly threatened by habitat loss and diseases. This highlights the importance of studying the genetics of a species before assessing its conservation status and creating management plans. By preserving the species and its habitat more amphibian species and the ecosystem services they provide will be protected. Conservation programs to improve the health of *A. moreletii* should be implemented, especially in Pacific and Veracruz populations. Water containers simulating ponds could be set within forest patches to give frogs more potential breeding sites. This could be a first step into supporting the populations to grow and to avoid low diversity levels and inbreeding. These types of programs have been very successful

for the *in-situ* conservation of *A. lemur* in Costa Rica (pers. comm Brian Kubiki 2016). Environmental education plans and projects involving the local communities are very important to have a successful outcome for the species.

One of the main causes of amphibian declines in Mesoamerica is infectious diseases like *B. dendrobatidis* (Lips *et al.*, 2005; Cheng *et al.*, 2011; Mendelson *et al.*, 2014; Scheele *et al.*, 2019). Research has focused on the amphibian skin microbiome since it acts as an immune system to protects the host (Harris *et al.*, 2009; Becker *et al.*, 2015). More and more microbiomes of different species and environments are being characterized to understand its dynamics with the host, environment and diseases (Flechas *et al.*, 2012; Bletz *et al.*, 2017; Bletz, *et al.*, 2017b; Abarca *et al.*, 2018; Ellison *et al.*, 2019). In chapter 4 the skin-bacterial communities of *A. moreletii* were characterized in several populations in Guatemala. On this species the microbiome is linked to the host and potentially to the microhabitat they inhabit. Populations occupying a degraded environment tend to have less diverse bacterial communities. This shows how the destruction and pollution of the environment can affect the host's microbiome and potentially cause health problems (McCoy and Peralta, 2018). A drastic reduction in the skin-bacterial diversity when individuals are infected with Bd was detected in all populations. This is a real concern for *A. moreletii* populations since it has been documented that the pathogen caused population declines in Mexico (Lips *et al.*, 2005). Mesoamerican amphibians were highly affected by Bd and it is crucial to keep monitoring amphibian populations and determine if this pattern could be found in other regional species.

In the last chapter I studied the effects that husbandry practices could have on the bacterial communities associated with the skin of *Bolitoglossa* salamanders and *Plectrohyla matudai*. Captive programs are a crucial part of amphibian conservation and have been the last result for several species around the world (Harding *et al.*, 2015; Lewis *et al.*, 2019). However, it is important to understand the changes that an individual can undergo during the different stages and protocols of an *ex-situ* program, especially if the aim is reintroduction. For the *Bolitoglossa* salamanders I found significant differences for the beta diversity during the different sampling stages. The composition and structure of the communities was disturbed mainly

during the quarantine stage. In the case of *P. matudai* significant differences were detected for alpha and beta diversity during the different sampling stages, mainly between field and quarantine, and quarantine and tanks. While comparing field and tanks I did not find any significant difference. These results indicate the importance of tank enrichment for the amphibian skin microbiome. Quarantine involved a simple plastic box with wet paper towels and it has been documented that sterile (Loudon *et al.*, 2014) or simpler environments (Michaels *et al.*, 2014) and stress of captivity and handling (Antwis *et al.*, 2014) can disrupt the bacterial communities. Some individuals that tested negative for Bd in the field, died during quarantine showing Bd symptoms, it is believed that the decrease in the bacterial community composition gave Bd an opportunity to colonize the host, and this has been observed in other species (Jani and Briggs, 2014). *P. matudai* has been identified as a surrogate species for *ex-situ* research by Amphibian Ark (Guatemalan conservation needs assessment workshop, 2010) and understanding more about its behaviour in captivity is important for the conservation of the genus, of which many species are endangered. As far as it is known this is the first time *Plectrohyla* frogs are kept in captivity.

Final conclusion and future work

Mesoamerican forests are disappearing at alarming rates, and it is important to document the diversity of the species living in them before they also disappear. Genetic and molecular biology tools are helping biologists to understand more about populations diversity and ecology. This project is a good example of the importance of integrating genetic and ecological data when doing conservation and species assessments. Following the results *A. moreletii* should be re-assessed to protect the species and its environment and to guaranty its survival. Conservation programs should be developed for the different ESUs especially in the Pacific Coast populations and the ones in Veracruz. The next step for *A. moreletii* conservation would be to work with landowners and communities to inform them about the importance of amphibians and start monitoring programs.

Equally important is to describe the microbiomes of different species and its interaction with pathogens to determine how the Bd can affect other species in the region, and as a way of monitoring the evolution of the pathogen in Central America. As well as starting more husbandry initiatives to understand how a captive environment can affect the local amphibian species, its behaviour and microbiome. This could lead to the creation of husbandry guidelines that could be useful in the case of sudden population declines. Finally, international collaborations between scientists from different Central American countries and Mexico for the creation of conservation and management programs are crucial to secure a future for Mesoamerican amphibians.

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