Assessing Diversity, Culturability and Context-dependent Function of the Amphibian Skin Microbiome

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ACADEMIC ABSTRACT

Emergent infectious diseases are a major driver of the accelerated rates of biodiversity loss that are being documented around the world. Global losses of amphibians provide evidence of this, especially those associated with chytridiomycosis, a lethal skin disease caused by the fungus *Batrachochytrium dendrobatidis* (Bd). Amphibian skin can harbor diverse bacterial communities that, in some cases, can inhibit the growth of Bd. Thus, there is interest in using skin bacteria as probiotics to mitigate Bd infections in amphibians. However, experiments testing this conservation approach have yielded mixed results, suggesting a lack of understanding about the ecology of these microbial communities. My dissertation research aimed to assess basic ecological questions in microbial ecology and to contribute to the development of probiotics using amphibian skin bacteria. First, to assess whether environmental conditions influence the function of amphibian skin bacterial communities, I conducted a field survey across low and high elevation populations of an amphibian host to assess their skin bacterial communities and metabolite profiles. I found that similar bacterial communities produced different metabolites at different locations, implying a potential functional plasticity. Second, since culturing is critical for characterizing bacteria, I aimed to identify the culture media (low vs high nutrient concentration) that recovers the most representative fraction of the amphibian skin bacterial community. I found that media with low nutrient concentrations cultured a higher diversity and recovered a more representative fraction of the diversity occurring on amphibian skin. I also determined that sampling more individuals is critical to maximize culture collections. Third, I assessed the diversity of the amphibian skin fungal community in relation to Bd infection across eight amphibian species. I determined that amphibian species was the most important predictor of fungal diversity and community structure, and that Bd infection did not have a strong impact. My dissertation highlights the importance of environmental conditions in the function of amphibian skin bacteria, expands our knowledge of the understudied fungal component of the amphibian skin microbiome, and complements current efforts in amphibian conservation.

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GENERAL AUDIENCE ABSTRACT

In light of the global losses of amphibian diversity due to, in part, the skin disease chytridiomycosis (caused by the fungus *Batrachochytrium dendrobatidis* [Bd]); the discovery that some amphibian-skin bacteria can inhibit Bd growth provides hope for amphibian conservation via their use as probiotics to control Bd infections. However, experiments testing these bacteria have yielded inconsistent results, suggesting a limited understanding about the factors influencing the diversity of amphibian-skin microbes and their ability to inhibit Bd. Also, efforts to identify effective candidates for probiotic therapy are still premature. Thus, my dissertation had an ecological emphasis and focused on complementing conservation efforts focused on probiotics. First, I assessed whether environmental conditions influence bacterially-produced products, which can have antifungal properties. Specifically, I surveyed low and high-elevation populations of an amphibian species to assess the skin-bacteria and their products. I determined that, while skin bacterial communities were similar across an environmental gradient, their products differed, suggesting potential different antifungal properties. Second, I assessed the ability of different culture media types (low vs high nutrient concentrations) to grow a high portion and most representative fraction of the amphibian-skin bacteria. I found that culture media with low nutrient concentrations allowed the growth of a higher diversity of the bacteria occurring on the amphibian-skin, including the abundant members, and also determined that including a large number of amphibians is the best way to improve culture collections. Third, I assessed the fungal diversity occurring in the skin of different amphibian species and how it might response to Bd infections, and examined whether skin-fungi interact with co-occurring bacteria. I found that the amphibian species was the most important driver of the fungal diversity, and that Bd infection did not influence the diversity of these communities. Moreover, I identified the most diverse fungal phyla occurring in the amphibian-skin and determined that these fungi might interact with co-occurring bacteria. My dissertation contributes to our understanding about the influence of the environmental conditions in the amphibian-skin bacteria, expands our limited knowledge on the amphibian-skin fungi, and complement current amphibian conservation efforts.

Dedication

To my parents and brothers

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Attributions

Chapter 2: Variation in Metabolite Profiles of Amphibian Skin Bacterial Communities Across Elevations in the Neotropics

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Chapter 3: Culture Media and Individual Hosts Affect the Recovery of Culturable Bacterial Diversity from Amphibian Skin

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Chapter 4: Amphibian skin fungal communities vary across host species and regions, but do not correlate with infection by a pathogenic fungus

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Chapter 1: General Introduction

Biodiversity crisis: The Case of Amphibians

The increasing rate of loss of many species and habitats has prompted scientists working in the field of conservation to talk about a "biodiversity crisis" (Collins et al., 2009). Some authors have concluded that the rate at which species are disappearing is "alarming" (Collins et al., 2009), with analyses examining the effects of tropical deforestation estimating the rate of extinction of species to be 1000 to 10,000 times higher than the rate in the fossil record (Wilson, 1992; Collins et al., 2009).

Among vertebrate groups, amphibians provide additional evidence of an existing biodiversity crisis and a potential sixth mass extinction (Collins, 2010; Wake and Vredenburg, 2008). For example, recent estimates suggest that current amphibian extinction rates are approximately 211 times higher than the background rates (Blaustein et al., 2012). Importantly, an analysis of the conservation status of amphibians concluded that worldwide 32% of all amphibian species are currently listed as Vulnerable, Endangered, or Critically Endangered in the IUCN Red List; and that 43% of amphibian species have populations experiencing declines (Stuart et al., 2004).

Threats to amphibian biodiversity: Effects of an emergent infectious disease

Six major threats, acting synergistically or alone, have been proposed as the causes of amphibian population declines and extinctions (Collins, 2010). These threats can be divided into historical and modern threats. Commercial use, introduced species and land use change are considered to be historical causes (Collins, 2010; Collins and Storfer, 2003), while climate change, contaminants and emerging infectious diseases are considered to be modern threats. These modern threats are also thought to be the main causes of the "enigmatic declines" of amphibians populations [i.e. population declines within protected areas] (Collins, 2010; Stuart et al., 2004).

Of all the threats to amphibian populations, one of the largest is the emerging infectious disease, chytridiomycosis, which is caused by the fungus *Batrachochytrium dendrobatidis* (hereafter Bd). Chytridiomycosis is responsible for many of the dramatic population declines and extinctions experienced by amphibians around the globe, including many enigmatic declines in protected areas (reviewed in Voyles et al., 2011), and is currently considered as the greatest threat to biodiversity by a disease in our times (Bletz et al., 2013; Wake and Vredenburg, 2008).

Bd infections and chytridiomycosis outbreaks are now recognized to be globally widespread. Chytridiomycosis was first determined as a direct cause of amphibian population declines in two distant locations, Panama and Australia (Berger et al., 1998), prior to the description of the fungal pathogen associated with this disease (Longcore et al., 1999). Importantly, this pathogenic fungus is currently known to exist in all the continents where amphibians exist, and to infect over 500 amphibian species from 54 countries (Fisher et al., 2009; 2012). However, the magnitude of chytridiomycosis outbreaks varies across regions of the world (Crawford et al., 2010). In terms of diversity, chytridiomycosis has a severe impact on local amphibian communities in some regions (Berger et al., 1998; Vredenburg et al., 2010). For example, in highly diverse areas such as the Neotropics, chytridiomycosis has caused extirpation of >50% of the amphibian species in a community, leaving the remaining species persisting at ~20% of their historic abundance (Lips et al., 2003). In addition, in temperate zones, chytridiomycosis has caused sharp declines in populations of fire salamanders (*Salamandra*

salamandra), common toads (*Bufo bufo*) and midwife toads (*Alytes obstetricans*) within a natural park in central Spain (Bosch and Martínez-Solano, 2006), where the latter species disappeared from 86% of its breeding habitats (Bosch et al., 2001). Lastly, another example from the temperate region is the mass mortalities and local extinctions experienced by yellow-legged frogs (*Rana muscosa* and *Rana sierrae*) following the arrival of Bd in three distinct metapopulations in California, USA (Vredenburg et al., 2010).

Consequences of such drastic biodiversity losses can result in ecosystem level effects (Blaustein et al., 2011). For example, sharp amphibian declines in neotropical highland streams affected algal community structure and therefore primary production, altered the dynamic of organic matter and contributed to population changes in aquatic insects and amphibian predators (Whiles et al., 2006). Such effects suggest that due to the different life histories and ecological roles of amphibian life stages in the ecosystem (e.g. aquatic herbivorous tadpoles, terrestrial carnivorous adults) losing one amphibian species is actually functionally equivalent to losing two species (Whiles et al., 2006).

Biology and ecology of Batrachochytrium dendrobatidis (Bd)

Overview of Bd life cycle and pathology

The pathogenic fungus Bd belongs to the phylum Chytridiomycota and possesses distinctive morphological characteristics that placed this species into a new monotypic genus when it was described (Longcore et al., 1999). However, a closely related, recently described chytrid fungus that primarily infects salamanders has now been placed within this genus as well, based on morphological features and molecular analyses (Martel et al., 2013). This new pathogenic fungus was named *Batrachochytrium salamandrivorans*, and is associated with mass mortalities and population declines of fire salamanders (*S. salamandra*) in the Netherlands (Sluijs, 2013). Together these two chytrid species represent the only two lineages within the phylum Chytridiomycota that are adapted to a vertebrate host, and that are pathogenic to amphibians (Martel et al., 2013).

Bd is an intracellular parasite that has two life stages: an aquatic, motile and short-lived zoospore, which represents the dispersal or infectious stage; and a stationary thallus that develops inside the host cell into a zoosporangium, within which the zoospores develop and are released via a discharge tube (Berger et al., 2005a). Infection by this pathogen takes place via zoospore colonization of the keratinized cells of amphibian epidermis in post-metamorphic stages (specifically within the cells of the *stratum granulosum* and *stratum corneum*) and mouthparts in tadpoles (Berger et al., 2005a; Longcore et al., 1999; Voyles et al., 2011). Up to three sporangia have been identified within a single host cell (Berger et al., 2005a; Voyles et al., 2011), suggesting that infection intensities can be very high in terms of zoospore production, which has been suggested to be a critical component for pathogenesis (Voyles et al., 2011; Vredenburg et al., 2010). Bd infection does not seem to be distributed randomly on the body of the amphibian, tending to be more intense in the ventral surfaces of the body and toes (Berger et al., 2005b).

Primary pathological signs of infection in hosts are hyperkeratosis (thickening of the *stratum corneum*), hyperplasia in the epidermis, and an increase in skin sloughing (reviewed in Voyles et al., 2011). Severely infected hosts present a series of behavioral and physiological clinical signs, including lethargy, lack of appetite, abnormal postures, cutaneous erythema, irregular skin sloughing and loss of righting reflex (Voyles et al., 2009; 2011). Bd infections disrupt vital physiological functions by reducing the functional capacity of amphibian skin,

where the transport of electrolytes is reduced by approximately 50%. This results in substantial decreases of potassium and sodium concentrations, which lead to cardiac arrest (Voyles et al., 2009). Furthermore, analyses regarding the pathophysiology of Bd on yellow-legged frogs during a chytridiomycosis outbreak supports the hypothesis that electrolytes and fluid imbalance are "critical" components of the pathology of this disease (Voyles et al., 2012b).

Bd-host interaction (amphibian immune responses)

Amphibians have complex immune systems with both an innate and acquired component (Bell et al., 2013; Voyles et al., 2011). However, a strong acquired immune response to Bd infection has not been observed in many species (Berger et al., 2005b; Voyles et al., 2011; Woodhams et al., 2007a). Similar to other pathogenic fungi that have the ability to evade host immune responses (Brown, 2011; Fites et al., 2013), Bd is able to produce toxic molecules that impair effective immune responses by inhibiting the proliferation of lymphocytes and inducing apoptosis (Fites et al., 2013). Yet, many aspects of the immune response to Bd infection are poorly understood (Venesky et al., 2014); research has focused on a limited range of immune responses, mainly on innate responses, as compared to acquired immune responses (Blaustein et al., 2012).

Evidence of an acquired immune response to Bd is not clear due to mixed results from different studies (Venesky et al., 2014), which suggests that research aiming to elucidate the role of acquired immunity is at an early stage (Bell et al., 2013; Ramsey et al., 2010; Richmond et al., 2009). For example, one study, using an immuno-genetic approach, documented reduced expression of the genes related to an adaptive response in experimentally infected frogs compare to non-infected ones in the species *Silurana* (*Xenopus*) *tropicalis* (Venesky et al., 2014). On the other hand, another study found an association between specific alleles in the locus IIB of the major histocompatibility complex (MHC) and survival after Bd exposure, and also that heterozygosity in this complex was a predictor of survival (Savage and Zamudio, 2011). In addition, there are also heterogeneous results regarding the response of immunized amphibians to subsequent Bd infection. While immunized *Xenopus laevis* presented an elevated number of Bd-specific antibodies following injection of heat-killed Bd compared to non-immunized individuals (Ramsey et al., 2010), another study that used a different immunization method (injection of formalin-killed Bd), found no differences in survival or resistance to Bd infection between immunized and non-immunized individuals of *Rana muscosa* (Stice and Briggs, 2010; Venesky et al., 2014). Stice and Briggs (2010) also monitored the survival of immunized individuals in the field, and suggest that vaccination might not be an effective mitigation method, and that a Bd vaccine might not be possible (Venesky et al., 2014).

As previously mentioned, the innate immune response is the most studied component of amphibian immunity to Bd, specifically the non-specific skin antimicrobial peptides (AMPs; Blaustein et al., 2012; Venesky et al., 2014; Voyles et al., 2011). AMPs can effectively inhibit Bd growth and prevent skin colonization by Bd, and given that they are produced by the epidermal granular glands, they might directly interact with Bd (Rollins-Smith, 2009). AMP activity against Bd has been observed for over 40 different types of peptides from approximately 27 amphibian species, where 83% of these peptides were effective at killing Bd in lab assays (reviewed in Rollins-Simth, 2009). Yet, *in vitro* studies have provided most of this evidence, which constrains the generalization of the effectiveness of this immune response against Bd *in vivo* (Voyles et al., 2011). However, skin peptide effectiveness *in vitro* against Bd does "correlate" with the population trends of some Australian frogs species after chytridiomycosis

outbreaks (Woodhams et al., 2005). In addition, in neotropical frogs, accurate predictions have been made regarding susceptibility to Bd infections based on the concentration of peptides collected (Woodhams et al., 2006). The mechanism via which skin antimicrobial peptides inhibit or prevent Bd infections remains unknown (Voyles et al., 2011); however, it is thought that skin peptides can disrupt the membrane of zoospores, which lack a cell wall, and penetrate the cell wall of zoosporangium, which have been killed within four days after interacting with skin peptides (reviewed in Rollins-Smith, 2009).

Another component of amphibian defenses against pathogens, such as Bd, are the symbiotic microbes associated with amphibian skin, which likely interact with the other components of amphibian defenses [e.g. skin antimicrobial peptides] (Myers et al., 2012; Woodhams et al., 2012) and pathogens. There is currently evidence that some members of these skin microbial communities are able to inhibit or reduce Bd growth, and possibly prevent infection in some amphibian hosts (e.g. Bell et al., 2013; Brucker et al., 2008a; 2008b; Harris et al., 2006; Lam et al., 2011).

Interactions of the skin microbiota with the innate immune system (e.g. skin antimicrobial peptides), have not been explored extensively (Myers et al., 2012). Yet, there are two independent studies that suggest a potential interaction between the skin microbial communities and the AMPs. Myers et al. (2012) determined that AMPs in *Rana muscosa* occurred at a reduced concentration, which allowed the colonization of the anti-Bd bacteria *Pseudomonas fluorescens* and production of its antifungal metabolite 2,4-DAPG to inhibit Bd growth *in vitro*. Another study showed this interaction from another perspective, where a reduction of the amphibian skin microbial community intensified the production of AMPs in response to Bd infection (Woodhams et al., 2012). These results imply that the presence of microbes reduces the production of costly AMPs and that some bacteria, potentially effective probiotics, can induce the production of AMPs through immunomodulation (reviewed in Bletz et al. 2013).

Amphibian skin microbial communities: ecology and evidence of anti-Bd properties

Overview of host-symbiotic microbe interactions

Metazoans serve as suitable habitat for many microorganisms, which has led to symbiotic relationships among these organisms (Belden and Harris, 2007). Within an evolutionary context, some authors have suggested that the host and their symbiotic communities as a whole should be considered as the unit of natural selection due to the variety of functions and fitness benefits associated with these microbes (Daskin and Alford, 2012; Rosenberg et al., 2007; 2010). As a result of the co-evolution of the host and its microbiota, in some cases the host depends on its symbiotic microbes for vital processes (Belden and Harris, 2007), including defense against pathogens, such as pathogenic fungi (Gil-Turnes et al., 1989; Kaltenpoth et al., 2005; Scherlach et al., 2013). Furthermore, the evolutionary history of symbiotic microbes with their respective hosts has led to host-species specific microbial communities. For example, McKenzie et al., (2011) determined that host species was a strong predictor of bacterial community similarity among amphibian species. Symbiotic microbes have vital functions and contribute to host survival and fitness. For example, microbes may assist in the breakdown of cellulose in the gut of termites (Belden and Harris, 2007; Wenzel et al., 2002), and bacteria can enhance bioluminescence in aquatic metazoans (Chun et al., 2008). In addition, the importance of the gut microbiota for human health and disease provides some of the most compelling evidence about how these microbes benefit their hosts (reviewed in Rosenberg et al., 2010).

Ecology and function of amphibian skin symbionts

Despite chytridiomycosis being widely recognized as a major threat to amphibian biodiversity, there is not a broadly effective treatment to mitigate this disease in wild populations (Becker et al., 2009; Daskin and Alford, 2012; Harris et al., 2009). However, hope has been generated by the recent discovery that bacteria associated with amphibian skin can inhibit the growth of Bd, and treating some amphibian species with these bacteria can reduce mortality (Daskin and Alford, 2012).

Culturable bacterial species from amphibian skin that can inhibit Bd growth have been identified from a broad range of bacterial genera (Woodhams et al., 2015). Among these genera, *Chryseobacterium*, *Janthinobacterium* and *Pseudomonas* have been the most conspicuous in terms of being identified in the most studies. Importantly, these three genera of bacteria have been identified on different amphibian species from different geographic locations, which include both temperate and tropical regions. Three bacterially-produced anti-fungal metabolites have been found to inhibit Bd growth or prevent Bd colonization. These metabolites are 2,4-diacetylphloroglucinol (2,4-DAPG), indole-3-carboxaldehyde (I3C) and violacein, which have been isolated from *Lysobacter gummosus,* in the case of 2,4-DAPG, and *Janthinobacterium lividum,* in the case of I3C and violacein (Brucker et al., 2008a; 2008b).

The evidence of inhibitory activity by bacteria associated with amphibian skin has been based mainly on laboratory experiments that involve *in vitro* challenge assays against Bd. Importantly, *in vitro* challenge assays have led to the identification of Bd inhibitory bacterial species, antifungal metabolites and to the observation that motile zoospores produced by Bd react to antifungal metabolites by chemotaxis (Bell et al., 2013; Brucker et al., 2008a; 2008b; Harris et al., 2006; Lam et al., 2011). In addition, the determination that Bd zoospores avoid or move away from substrates with antifungal metabolites has been suggested as a potential mechanism by which some amphibians can harbor non-lethal infection levels due to, in part, reduced re-infection probabilities (Lam et al., 2011). Furthermore, bioaugmentation of anti-Bd bacteria on amphibian hosts has been conducted, and the results suggest that for some amphibian species, probiotic treatment may reduce morbidity and mortality associated with Bd exposure (Becker et al., 2009; Harris et al., 2009b; 2009a). Yet, the heterogeneous results obtained from the few *in vivo* studies attempting to use bioaugmentation of a probiotic to prevent Bd infection reflect the gaps in the current literature and the need to devote more research effort in this area. While some studies have had success in terms of producing successful colonization of amphibian skin and inhibiting or reducing Bd infection levels in both laboratory and field conditions (reviewed in Bletz et al., 2013), others have been unsuccessful (Becker et al., 2011).

Amphibian skin microbial communities may also contribute to the variation in chytridiomycosis outbreak intensity observed in wild populations. For example, two independent studies found an association between a high proportion (~80%) of individuals with anti-Bd bacteria in populations of highly susceptible species (*Rana muscosa* and *Rana sierrae*) and the co-existence with Bd several years after the arrival of the pathogen (Lam et al., 2010; Woodhams et al., 2007b). In addition, Lam et al. (2010) predicted the persistence of a Bd-naïve population of *Rana muscosa* based on the proportion of individuals with protective bacteria. This population survived the chytridiomycosis outbreak and currently persists with endemic Bd. There may be a threshold for protection in terms of the proportion of individuals with protective bacteria, above which an epizootic infectious stage of an emergent infectious disease will change to an enzootic stage (Bletz et al., 2013; Lam et al., 2010; Woodhams et al., 2007b). However, this association is restricted to populations of two closely related species in the temperate zone.

In contrast to the large amount of research that has focused on the amphibian skin bacteria, studies characterizing and assessing the function of the fungal communities associated with the amphibian skin are still scarce and lagging behind those on bacteria, despite fungal organisms being highly abundant in subadults and adults (Kueneman et al., 2015). In addition, recent work determined a potential defensive role against Bd by the amphibian skin fungal communities, and suggested that they might be good candidates for the development of probiotics because their application did not induce an immune response by the amphibian host (Kearns et al., 2017).

The skin, including amphibian skin, is a dynamic ecosystem influenced by the host and environmental factors that shape colonization and resilience of microbes (Costello et al., 2009; Kueneman et al., 2013; Rosenthal et al., 2011). Importantly, an understanding of the ecology of these microbial communities will substantially complement the basis for the development of mitigation techniques based on using anti-Bd microbial species as probiotics (Kueneman et al., 2013).

Understanding the effect of environmental factors on shaping the diversity, structure and function of these symbiotic microbial communities is an underexplored research area that is important in light of global environmental change (Belden and Harris, 2007; Kueneman et al., 2013). For instance, there are currently few studies that address how the environment influences the function of the amphibian skin microbes (Daskin and Alford, 2012). In addition, other critical aspects of the ecology of these microbial communities that are relevant to conservation remain poorly understood, for example, whether the function, including defensive function, changes with fluctuations in the microbial community structure (Bletz et al., 2017; Fierer et al., 2012). The development of culture-independent techniques has allowed for the identification of un-culturable bacteria, and has provided scientists with a more accurate perspective of the structure of these communities, and with a better understanding about some of the most important factors influencing them (Caporaso et al., 2011; 2012; Costello et al., 2009; Dethlefsen et al., 2007).

Overview of dissertation research

In Chapters 2 – 4, I aimed to contribute to our current understanding of the ecology of the microbial communities associated with amphibian skin, and to complement current efforts to develop an amphibian conservation strategy based on the use of amphibian skin bacteria as probiotics to mitigate Bd infections. Within this context, in **Chapter 2**, I examined the effect of environmental conditions on the relationship between the structure and function of amphibian skin bacterial communities. Specifically, I conducted a field survey in Panamá, where Bd outbreaks have been most severe at high elevations, to assess the skin bacterial communities and metabolite profiles of the species *Silverstoneia flotator* from three high- and three low-elevation populations representing a range of environmental conditions (Medina et al., 2017a). To contribute more directly to conservation efforts, in **Chapter 3**, I conducted a comparative study to assess different culture media types (high vs. low nutrient concentration) for culturing amphibian skin bacteria, and to determine what culture media can recover the most representative fraction of the bacterial community relative to a culture-independent method. In addition, I determined whether the bacterial communities growing on the different media types differ in their ability to inhibit Bd growth using *in vitro* challenge assays (Medina et al., 2017b). Studies characterizing and assessing the fungal communities associated with amphibian skin are still scarce and lagging behind those on bacteria. Therefore, in **Chapter 4**, I assessed the diversity and natural variation of the skin fungal communities across eight species of

amphibians, representing two distinct regions (temperate and tropical) and different life-histories. In addition, I assessed whether infections by Bd influence the diversity and community structure of these communities, and examined potential patterns of co-occurrence between the composition of the bacterial and fungal communities associated with the amphibian skin. Lastly, **Chapter 5** provides a general conclusion of the results from my dissertation research and includes recommendations for future research on the amphibian-associated microbiome and microbial ecology in general.

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Chapter 2: Variation in Metabolite Profiles of Amphibian Skin Bacterial Communities Across Elevations in the Neotropics

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Abstract

Both the structure and function of host-associated microbial communities are potentially impacted by environmental conditions, just as the outcomes of many free-living species interactions are context-dependent. Many amphibian populations have declined around the globe due to the fungal skin pathogen, *Batrachochytrium dendrobatidis* (Bd), but enivronmental conditions may influence disease dynamics. For instance, in Panamá, the most severe Bd outbreaks have occurred at high elevation sites. Some amphibian species harbor bacterial skin communities that can inhibit the growth of Bd, and therefore, there is interest in understanding whether environmental context could also alter these host-associated microbial communities in a way that might ultimately impact Bd dynamics. In a field survey in Panamá, we assessed skin bacterial communities (16S rRNA amplicon sequencing) and metabolite profiles (HPLIC-UV/Vis) of *Silverstoneia flotator* from three high and three low elevation populations representing a range of environmental conditions. Across elevations, frogs had similar skin bacterial communities, although one lowland site appeared to differ. Interestingly, we found that bacterial richness decreased from west to east, coinciding with the direction of Bd spread through Panamá. Moreover, metabolite profiles suggested potential functional variation among frog populations and between elevations. While the frogs have similar bacterial community structure, the local environment might shape the metabolite profiles. Ultimately, host-associated community structure and function could be dependent on environmental conditions, which could ultimately influence host disease susceptibility across sites.

Introduction

Host-associated bacteria play key roles in development, reproduction, digestion and protection against pathogens for their hosts [1]. In recent decades, culture-independent molecular techniques have rapidly expanded our knowledge of the diversity, ubiquity and functional ability of these symbiotic communities. In addition, the integration of different molecular techniques and "omics" methods, such as metabolomics combined with 16S rRNA gene amplicon sequencing, are providing an opportunity to address important ecological questions, including insights into the potential link between community structure and function in complex microbial communities [2, 3]. For example, the use of 16S rRNA gene amplicon sequencing has provided important insights into the structure-function relationship in soil microbial communities, where functional redundancy might be present in important soil microbial processes [4], and in the gut microbiome of mammals, in which functional repertoires of microbiome genes can be predicted by the structure of the bacterial community [5].

One key function of host-associated microbes on animal hosts is host defense against pathogens, which has been observed across distantly related host taxa, including corals, shrimp, *Hydra*, insects, birds, humans, and amphibians [6-11]. For example, amphibians harbor diverse skin bacterial communities that appear to be host-species specific [12-15]. Some of these skin bacteria can protect their amphibian hosts from the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd) [15-19], seemingly through the production of secondary metabolites that inhibit Bd growth [18, 20, 21]. As Bd has been associated with drastic global amphibian population declines [22] and infects over 500 amphibian species across the globe [23], interest has grown in understanding the factors that shape these microbial communities and their function.

In general, relatively little is known about how environmental factors influence the structure and function of host-associated microbial communities, although there is certainly appreciation more broadly for the role of context-dependency in determining the outcome of symbiotic interactions [24-26]. For example, for host-pathogen interactions, changes in temperature can alter transmission rates of pathogenic fungi from adult bark beetles to pine trees [27], enhance bleaching in corals [28, 29] and influence the morbidity of white-nose syndrome in bats [30]. Chytridiomycosis outbreaks in amphibians, caused by Bd infections, have varied in intensity across space and time, which suggests a context-dependency in the outcome of interactions among the amphibian host, Bd and the environment [31, 32]. In the Neotropics, amphibian diversity losses due to Bd have been substantial [33], but in general Bd-induced population declines and local extinctions have been more dramatic at higher elevations relative to the lowlands [34-37], potentially because of variation in environmental factors (e.g. temperature and rainfall) that alter disease outcomes.

Indeed, context-dependent outcomes of host-microbiome-pathogen interactions could play a critical role in wildlife diseases [32]. But while some effort has focused on understanding how amphibian-Bd interactions might change across elevations (e.g. [38-42], little work has addressed changes in the amphibian-microbiome interaction with elevation. In the present study, we aimed to determine how the diversity, structure, and potential function (represented by metabolite profiles) of skin bacterial communities on amphibians vary with elevation. Specifically, we sampled populations of the species *Silverstoneia flotator* – a species thought to be tolerant to Bd and with a broad distributional range at both highland and lowland sites. We hypothesized that we would observe distinct bacterial communities and metabolite profiles across elevations given that abiotic factors, such as precipitation, temperature ranges and strength of seasonality, vary along elevational gradients. Importantly, these environmental factors could directly or inderectly influence the skin bacterial communities.

Materials and Methods

Silverstoneia flotator (Family: Dendrobatidae, Dunn 1931) occurs from '5-1050m' in Panamá [43]. Across this elevational range, vegetation changes from lowland wet/moist forest (<600 m) to premontane forest (600-1500 m), which is associated with changes in climatic factors such as temperature and precipitation [43]. *S. flotator* is a common terrestrial diurnal species occurring in leaf litter, including along streams. This species is broadly distributed across Panamá, and may be tolerant of Bd infection considering it has not declined following Bd arrival at one highland site [33], and that there are populations persisting with Bd at other highland sites [36, 42].

Sample collection

In 2013, we sampled 10-15 *S. flotator* at each of six sites where Bd is known to occur to assess their skin bacterial communities, skin metabolites profiles and Bd infection status. Of the six sites, three were highland sites above 600 m, while the other three were lowland sites below 300 m (Table 1; Fig. 1). Among the study sites, chytridiomycosis has caused amphibian declines at two of the highland sites, El Cope and Cerro Campana [34, 36], while there have been no declines at one of the lowland sites, Soberanía, despite the presence of Bd [36]. There are no published studies for the other three sites describing the impact of Bd arrival on the amphibian community, though it is known that Bd is present at these sites (R. Ibáñez and J. Voyles pers. comm.).

During sampling, we handled each frog with a new pair of gloves and placed it in a sterile Whirl-Pak® bag (Nasc, Fort Atkinson, WI, USA) until being swabbed within 30 minutes. The swabbing protocol was conducted as in Belden et al. [14]; prior to swabbing, each frog was rinsed with 50 mL of sterile deionized water to remove any dirt and transient bacteria [12, 14, 44]. Then each frog was swabbed twice: the first swab was used to assess the skin bacterial community and Bd infection status, and the second swab was used to assess the metabolite profile. The first swab was a sterile rayon swab (MW113, Medical Wire Equipment), and the second swab was a pre-treated polyurethane swab (14-960-3J, Fisher Scientific). The polyurethane swabs were pre-treated to remove methanol-soluble impurities by stirring them in a methanol wash and letting them air-dry in a fume hood [45]. Each swab collection consisted of 20 strokes on the ventral surface, and 5 strokes in a single direction on each thigh and hind foot for a total of 40 strokes per swab. The swabs were individually placed in 1.5 mL centrifuge tubes and stored either in a cooler with dry ice or ice packs (samples from Soberanía, Cerro Azul and Sierra Llorona) or in liquid nitrogen (Cope, LowCope and Campana) during fieldwork. Temperature variation in short-term sample storage does not influence the assessment of bacterial community composition [46]. In the laboratory, swabs were placed at -80ºC until processing. Frogs were returned to the site of capture after swabbing.

Data from 60 frogs (N= 5-12/site) were used for the comparative analyses of the skin bacterial communities (Table 1). We expected to find a low incidence of Bd infection on *S. flotator* since it persists in sites with Bd and because we sampled during the beginning of the rainy season, which could be a time of low seasonal prevalence of infection in the neotropics (e.g. data from Costa Rica,[47]). Of the 60 frogs we swabbed, we preliminarily selected 40 individuals from four sites (two highlands: Cope High and Cerro Azul; and two lowlands: LowCope and Soberanía) to assess Bd infection status. For the metabolite profiles, we included a total of 72 individuals from five sites (N = 12-15/site, with no metabolite swabs from Sierra Llorona; Table 1). Out of these 72 individuals, 55 overlapped with the set of 60 used for the bacterial community analyses.

Sample and Data Processing

Skin bacterial communities

DNA was extracted from the first swab using the DNeasy Blood and Tissue kit (Qiagen, Valencia CA, USA) following the manufacturer's protocol, and with an initial lysozyme incubation at 37 ºC for 1 h. A final volume of 100 µL of DNA was obtained, which was used as the template DNA for the Bd infection analyses as well.

Skin bacterial communities were assessed by amplifying and sequencing the V4 region of the 16S rRNA gene with the primers 515F and 806R [48]. The barcoded reverse primer contained a 12-base error-correcting Golay code to tag PCR products, which allowed for multiplexing. PCR reactions (25 µL) were prepared with 12 µL of molecular grade PCR water, 10 µL of 5 Prime Hot Master Mix, 0.5 µL of each primer (10 µM concentration) and 2.0 µL of template DNA. PCRs were run in triplicate with a control that did not contain template DNA. PCR reactions were conducted with an initial cycle of 3 min. at 94 ºC, followed by 35 cycles of 45 s at 94 ºC, 60 sec. at 50 ºC, and 90 s at 72 ºC, with a final extension of 10 min. at 72 ºC. PCR products from triplicate reactions were pooled and visualized on a 1% agarose gel, and quantified using a Qubit 2.0 flourometer (Invitrogen, Carlsbad, California). PCR products of each sample (200 ng/sample; N=60) were pooled to make a composite sample and cleaned with the QIAquick PCR Purification Kit (Qiagen, Valencia, California). The final pooled sample was sent for sequencing on an Illumina Mi-Seq instrument with a 250 bp paired-end strategy at the Dana-Farber Cancer Institute of Harvard University following Caporaso et al. [48].

Raw sequences from the Illumina 16S rRNA gene amplicon sequencing were assembled with Fastq-join [49]. Assembled sequences were processed and quality-filtered using the default parameters of the Quantitative Insight Into Microbial Ecology (QIIME) pipeline [50], with the exceptions that we allowed for no errors in the barcodes, increased the number of minimum consecutive low quality base calls allowed before truncating a read (r) to 10, and decreased the fraction of the minimum number of consecutive high quality base calls to include a read (p) to 0.5. Sequences were clustered into operational taxonomic units (OTUs, ~bacterial species) based on a 97% similarity threshold using the UCLUST method [51], and OTUs were represented by the most abundant sequence. The representative sequences were aligned to the Greengenes 13_5 reference database [52] using PyNAST [53] and assigned taxonomy using the RDP classifier [54]. We removed OTUs with fewer than 0.001% of the total number of sequences [55]. The sequencing depth per sample ranged from 6132 – 96126, so we rarefied the dataset at a depth of 6000 reads/sample. The final dataset consisted of 3497 OTUs across the 60 samples, with 193-1079 OTUs (mean = 491±186) per frog.

Skin metabolites profiles

To assess the metabolite profiles, we isolated and characterized small molecules from the swabs using HPLC-UV/Vis detection [45]. This method is useful for identifying metabolite patterns across environmental gradients, even though we cannot identify individual compounds. Given the nature of the method, highly polar compounds, such as peptides and proteins that might be produced by the amphibian host, are not detected; thus, the compounds detected represented small molecules hypothesized to be bacterial secondary metabolites [45]. For the characterization of the metabolite profiles, swabs were sent frozen to Villanova University and processed as detailed in Belden et al. [14].

Compounds eluted from samples were characterized by retention times and UV-Vis chromophore. Retention times of all detected compounds (peaks) were normalized to that of a naphthalene internal standard (20.69 min). The retention time of each chromatographic feature was determined manually using Applied Biosystems Analyst software V.1.5.1. The generated dataset was then revised to account for slight variations in retention time across multiple samples, with a focus on major chemical components. Compounds present in multiple samples that displayed both similar retention times and identical spectroscopic features were assigned as a single compound, and unique compounds only detected in a single sample were considered

noise and therefore disregarded. Lastly, chromatographic features with less than 3,000 mAU x min were considered minor components and disregarded.

Bd detection and quantification

The 40 samples assessed for Bd were analyzed with a Taqman real-time PCR (qPCR) assay developed by Boyle et al. [56]. We amplified ITS-1-5.8S of the Bd genome using primers ITS1-3 Chytr and 5.8S Chytr and the probe MGB2. DNA standards were prepared by making serial dilutions for 1,000 – 0.1 zoospores genomic equivalents of the strain JEL 310, a strain isolated from *Smilisca phaeota* from Fortuna, Panamá [57]. Given that the copy number of the region ITS-1-5.8S of the strain used for the standards is unknown, a low value equal to one copy number could not be estimated, as described by Rebollar et al. [37]. Thus, a sample was considered infected when the values estimated by the qPCR assay were above 0.1 zoospores genomic equivalents. The samples were run in duplicate and considered positive when amplification was observed in both replicates. The infection intensity (i.e. number of zoospores equivalents) of a sample represents the average of both replicates.

Data Analysis and Statistical Methods

Overview

We assessed whether there were differences in skin bacterial community structure, metabolite profiles and structure-function relationships between lowland and highland populations of *S. flotator*. As there was very little Bd infection detected, we were not able to assess variation based on Bd infection intensity or prevalence. We also described the core microbiome to provide information about the bacterial taxa associated with a neotropical species that has survived chytridiomycosis epidemics and persists at sites with endemic Bd. Unless noted, all analyses were conducted in R version 3.2.2 [58].

Alpha and beta diversity analyses

Alpha diversity comparisons of skin bacterial communities between elevations and across sites were conducted for richness (OTUs/individual), phylogenetic diversity and the Shannon Index (*H'*, which assessed community evenness). These metrics were computed for each frog with QIIME. We compared alpha diversity estimates between elevations by fitting the metrics to generalized linear mixed models (GLMM), in which the variable "Site" was included in the models as a random effect to account for the nestedness of individual frogs within each site. Among site comparisons were performed by fitting the metrics to generalized linear models (GLM). For richness estimates, we used a negative binomial error distribution with the log link function to account for overdispersion; for elevation comparisons, we used the function glmer.nb from the package lmer4 [59], and for among site comparisons we used the function glm.nb from the package MASS [60]. For Shannon and phylogenetic diversity estimates we used a Gamma error distribution with the inverse link function to account for heteroskedasticity; for elevation and among site comparisons we used the functions glmer and glm from the package lmer4 [59], respectively. Multiple comparisons were conducted with Tukey tests using the function glht of the package *multcomp* [61], which includes multiple comparisons for generalized linear models. Moreover, we also compared metabolite richness (metabolites/frog) between elevations and among sites by fitting the metric to a linear mixed model (function lmer) and a linear model (function lm), respectively (packages lmer4 and stats). Visual assessments of residual plots with model predictions corroborated that these approaches were appropriate.

We performed GLMs to assess the effect of longitude (converted to UTM) on the alpha diversity estimates. GLMs were performed as described above, with a negative binomial error distribution for richness estimates, and a Gamma error distribution for Shannon and phylogenetic diversity. Visual assessments of residual plots with model predictions corroborated that these approaches were appropriate.

To examine whether elevation and site explained variation in the structure of the bacterial communities and metabolite profiles (beta diversity), we performed permutational multivariate analyses of variance, PERMANOVA [62], based on a Bray-Curtis dissimiliarity matrix for the OTUs and on a Jaccard dissimilarity matrix for the metabolites. Jaccard dissimilarities, which only take into account presence-absence, were used for the metabolite data because the HPLC-UV/Vis output does not allow for the estimation of relative abundance. Results were visualized using nonmetric multi-dimensional scaling (NMDS). The distance matrices were calculated with the function vegdist, and PERMANOVAS were performed using the function adonis in the *vegan* package [63].

Examining links between bacterial community structure and metabolite profiles

To assess potential relationships between bacterial community structure and metabolite profiles, we conducted a Mantel test. The Mantel test requires that the matrices being tested have the same samples, so we removed the 12 frogs from the metabolite dataset that were not represented in the OTU dataset. This resulted in the same 55 samples in both data matrices. We conducted the Mantel test in two ways: separately by individual sites, and then with all the sites together. Mantel tests were performed with the function mantel in the *vegan* package [63].

Incidence of Bd infections

Bd was only detected in one of the highland sites (Cope), and in only three frogs, so no comparisons regarding Bd across sites or elevation could be conducted. Bd prevalence and infection intensity at Cope (zoospore equivalents) was estimated using the Wilson interval and a bootstrap approach (BCa; 10000 permutations), respectively, using R [37]. As only three of the 40 frogs analyzed were Bd-positive, we did not quantify Bd in the remaining 20 frogs from the other two sites.

Core microbiome

The core microbiome is defined as “organisms common across microbiomes” [64], and may be fundamental to community function [64]. The core microbiome for *S. flotator* was determined using the compute_core_microbiome script in QIIME and was defined as OTUs present on ≥ 90% of all the frogs. The relative abundances of the core OTUs, across frogs, sites and elevations was visualized via a heatmap, using the function heatmap.2 in the package *gplots* [65]. We examined whether the relative abundance of each core OTU significantly differed based on elevation and site with a Kruskal-Wallis test. We calculated the corrected p-values for the multiple comparisons based on the Bonferroni procedure using the group_significance script in QIIME.

Results

Bacterial Community: Diversity and Structure

None of the three metrics of alpha diversity of bacterial communities differed between high and low elevations (richness: $\chi^2 = 0.06$, $p = 0.81$; Shannon: $\chi^2 = 0.01$, $p = 0.92$; phylogenetic

diversity: $\chi^2 = 0.24$, $p = 0.62$, Table S1a). However, we observed significant differences among sites for all the metrics (richness: deviance = 60.62, $p < 0.001$; Shannon: $F = 10.36$, $p < 0.001$; phylogenetic diversity: $F = 5.2$, $p < 0.001$). Alpha diversity pair-wise comparisons among sites showed a potential west to east trend, especially for richness and phylogenetic diversity estimates, where the sites west of the Panamá Canal had significantly higher diversity estimates than those on the east side (Fig. 2; Table S1b). The generalized linear models used to assess this pattern suggested a significant decline towards the east in the three alpha diversity metrics (richness: log-scale slope = -3.88E-06, SE = 7.27E-07, $p = <0.001$; Shannon: inverse-log scale slope = 5.55E-07, SE = 1.07E-07, $p = <0.001$; phylogenetic diversity: inverse transformed slope = 7.66E-08, SE= 1.67E-08, $p = <0.001$; Table S2, Fig. S1).

Bacterial community structure (beta-diversity) differed between elevations and among sites when taking into account all the sites (Fig. 3a,b. NMDS stress: 0.16; by elevation: adonis pseudo-$F = 5.83$, $p < 0.001$, $R^2 = 0.09$; by site: adonis pseudo-$F = 5.13$, $p < 0.001$, $R^2 = 0.32$). However, the differences in community structure and the variation explained by the factors 'elevation' and 'site' were likely driven by the distinct communities at LowCope, as shown on the NMDS. When LowCope was removed from the analysis, there was not a significant difference based on elevation or site for the remaining sites (by elevation: adonis pseudo-$F = 1.03$, $p = 0.34$, $R^2 = 0.021$; by site: adonis pseudo-$F = 1.53$, $p = 0.12$, $R^2 = 0.12$).

Metabolite Profiles: Diversity and Structure

A total of 84 unique metabolites were identified (range 25-38/frog; mean ± SD 33.38 ± 2.52). Metabolite richness differed significantly between elevations, although the means were very similar ($\chi^2 = 5.73$, $p = 0.017$; mean ± SD: high 33 ± 2.72; low 34 ± 1.97). There was no difference in metabolite richness among sites ($F = 1.48$, $p = 0.217$; mean ± SD: Campana 33 ± 2.47; Cerro Azul 33 ± 3.19; Cope 33 ± 2.73; CopeLow 34 ± 1.90; Soberanía 34 ± 2.11). Metabolite profiles differed between elevations and across sites (Fig. 4a,b; NMDS stress: 0.14; by elevation, adonis pseudo-$F = 10.37$, $p < 0.001$, $R^2 = 0.13$; by sites, adonis pseudo-$F = 15.53$, $p < 0.001$, $R^2 = 0.48$); however, site explained more of the observed variation (PERMANOVA: site $R^2 = 0.48$; elevation $R^2 = 0.13$). As one site (i.e. Soberanía) appeared to potentially drive this difference based on the NMDS ordination (Fig. 4b), we conducted a further analysis excluding the site 'Soberanía'. However, there was still significant variation in metabolite profiles among sites after excluding 'Soberanía' (adonis seudo-$F = 4.42$, $p < 0.001$, $R^2 = 0.20$, Table S3). All pairwise comparisons of metabolite profiles among sites were significantly different, except for LowCope-Campana (Table S3).

Linking Bacterial Community Structure and Metabolite Profiles

Based on Mantel tests, we found no correlation between the structure of the bacterial communities at the sites and their respective metabolites profiles (Campana: Mantel r statistic = -0.12, $p = 0.65$; Cerro Azul: 0.04, $p = 0.34$; Cope: 0.20, $p = 0.16$; LowCope: 0.03, $p = 0.36$; Soberanía: 0.11, $p = 0.31$; all sites together: 0.06, $p = 0.16$).

Bd Occurrence

Prevalence of infection by Bd at Cope was 30% (3/10 frogs; CI 95% 11-60%) and the mean infection load per frog was 250 (CI 95% 98-347) zoospore genomic equivalents. No Bd was detected at the other three sites we tested.

Core Microbiome

Despite a total of 3497 OTUs identified on *S. flotator*, only 25 OTUs were in the 90% core microbiome (from nine families in three phyla: Actinobacteria, Firmicutes and Proteobacteria; Table S4). The most abundant core OTUs (relative abundance ≥ 1%) were in the families: Cellulomanadaceae (mean relative abundance of family 15%), Comamonadaceae (1%), Enterobacteriaceae (1%), Pseudomonadaceae (4%) and Xanthomonadaceae (1%). There was no significant difference in the relative abundance of any core OTUs between elevations (Fig. 5), except for one Comamonadaceae (ID number 4456068) that had higher mean relative abundance ($\chi^2 = 12.62$, $p = 0.010$) at high elevation (0.015 vs. 0.008%). However, there was significant variation across sites for ten core OTUs (Fig. 5). This variation mainly reflects the difference at LowCope; six of the ten core OTUs occurred at a lower relative abundance there and two Enterobacteriaceae (ID numbers 2119418 and 814442) and one Pseudomonadaceae (269930) occurred at a higher relative abundance (Enterobacteriaceae ID 2119418: $\chi^2 = 21.68$, $p = 0.02$; Enterobacteriaceae ID 814442: $\chi^2 = 21.52$, $p = 0.02$; Pseudomonadaceae ID 269930: $\chi^2 = 23.19$, $p = 0.008$).

Discussion

In our study, we used replication of highland and lowland sites to try to understand the potential role of environmental variation in the structure and function of skin bacterial communities. However, we did not see key differences in bacterial communities on *S. flotator* between high and low elevations, either in terms of alpha diversity metrics or in terms of bacterial community structure. Skin metabolite profiles, which we used as an estimate of microbial community function, did differ between elevations. This suggests that environmental conditions at high and low elevation sites might not contribute to the establishment of completely different bacterial communities, but they could result in a change in function of the communities. While we cannot, with our current method, identify the specific metabolites that differed across elevations or among sites, this is a promising area of future research. In addition, the absence of an elevational pattern in the diversity of these amphibian-associated bacterial communities seems to be somewhat consistent with observations of free-living soil bacterial communities, where no consistent elevational patterns have been observed. For example, one study showed a negative relationship between elevation and diversity within the phyla Acidobacteria in the western USA [66], another one found a peak in OTU richness at mid-elevation at Mount Fuji in Japan [67], and lastly, a study conducted in the Andean region showed no elevational trend in the bacterial communities from organic and mineral soils [68]. Additionally, although an understanding of the variation in function of animal-associated bacterial communities across elevations is scarce, in free-living soil microbial communities changes in environmental factors across elevations were associated with changes in community function, therefore influencing microbe-mediated nitrogen dynamics [69].

While the structure of bacterial communities on *S. flotator* was also relatively consistent among sites, with the exception of LowCope, there was variation across sites in metabolite profiles. In addition, the bacterial communities and metabolite profiles were not correlated either within elevations or across sites. Taken together, these results suggest that similar communities may produce different combinations of secondary metabolites at different sites, which could infer a potential functional plasticity in these bacterial communities. This could be mediated by differences in environmental conditions among sites. For instance, Daskin et al. [70] determined that specific bacterial isolates cultivated at a range of temperatures ecologically relevant to the

amphibian-Bd interaction produce different quantities of the same metabolite and/or different types of metabolites. Moreover, Woodhams et al. [71] found variation in antifungal activity of metabolites against Bd from a bacterial isolate cultivated across a range of temperatures. Temperature is one of the main factors influencing bacterial cell growth and metabolic activity [72] and can influence the production of antibiotic compounds in bacteria [73, 74], potentially because of variation in temperature thresholds for the expression of genes involved in metabolite production [70, 75].

Understanding the link between biodiversity and ecosystem function can be challenging. Distinct approaches–studies across gradients and diversity manipulation–often give inconsistent results, including with bacterial systems [76]. Nevertheless, it seems likely that the link between bacterial community structure and function across ecological systems may be influenced by environmental conditions, including both abiotic and biotic factors. For example, the diversity of free living soil ammonia- and nitrite-oxidizing bacteria changes across gradients of inorganic nitrogen availability, which influences the nitrification process [76-78]. Also, pathogen protection of corals by coral-associated bacteria is influenced by temperature [28]. In our system, it is possible that variation in environmental factors at different elevations, such as temperature, has more of an effect on bacterial community function (metabolite profiles) than on the structure of the communitites. Biotic factors that can impact host-associated microbial communities, in particular, include potential host pathogens. We could not examine the link between current pathogen infection with Bd and skin bacterial community structure or function in the present study due to the low incidence of Bd in our samples. However, prior experimental studies in other amphibian host species suggest that the bacterial community on the skin can potentially alter the disease outcome of Bd exposure, and that Bd exposure can drive changes in the bacterial community structure [14, 57, 79-81]. Thus, potential context-dependency driven by biotic factors, such as pathogen presence, might add another layer of complexity in understanding environmental context-dependent outcomes between microbes and their host and the mechanisms driving community function in these systems.

While we could not examine the effect of current Bd infection on the microbiome in our study, we did observe a longitudinal trend in alpha diversity of the skin bacterial communities across our sites that is consistent with the west to east spread of Bd in Panamá [36, 37, 82, 83]. Richness and phylogenetic diversity, in particular, decreased from the western sites to the eastern sites. Two prior studies have examined changes in the skin bacterial communities across host populations with different Bd histories in Panamá. Rebollar et al. [84] found that the terrestrial species *Craugastor fitzingeri* had lower skin bacterial community diversity in populations where Bd occurs at an endemic infection stage compared to Bd-naïve populations, and higher relative abundance of some OTUs from the genera *Cellulomonas*, *Sanguibacter* and *Pseudomonas* in the populations where Bd is endemic. However, in a separate study, across a gradient of Bd endemic sites, no clear west-east pattern was identified in the skin bacterial communities and metabolite profiles of two pond-breeding frogs, *Agalychnis callidryas* and *Dendropsophus ebraccatus* [14]. It may be that there are host species-specific patterns in the response of the skin microbiota to the presence of Bd in the environment. Species-specific responses of skin bacterial communities to Bd might add complexity to the studies addressing the ecological processes shaping the composition, structure and function of these communities. This could partially explain the variable results regarding skin bacterial communities across Bd exposure experiments and across field studies working on distinct species [14, 57, 79-81].

We found that a very small fraction (0.7%) of the OTUs found on *S. flotator* can be considered as members of the core microbiome. But overall, the community was similar to what has been seen previously in Panamanian amphibians [14]. The most abundant OTUs identified in the *S. flotator* core belong to the genera *Cellulomonas* and *Pseudomonas*, which have also been found in high relative abundance on *Craugastor fitzingeri* occurring at sites in Panamá where Bd is endemic [84]. Moreover, the families Comamonadaceae and Pseudomonadaceae, both abundant in the core microbiome of *S. flotator*, include many anti-Bd isolates from non-Bd-susceptible Panamanian amphibian species [15]; and one OTU from the family Comamonadaceae was associated with individuals of the endangered species *Atelopus zeteki* that cleared experimental Bd infections [57]. Two core OTUs from *S. flotator* were in the family Enterobacteriaceae and were significantly more abundant in the Cope lowlands (LowCope), the only site with distinct bacterial communities relative to other sites. Interestingly, unlike the other study sites, the LowCope site was located in the proximity of a village. Importantly, there is evidence that amphibians and reptiles living on aquatic environments exposed to human domestic and industrial waste can harbor Enterobacteriaceae with high resistance against antibiotics and heavy metals, including the genus *Citrobacter* [85], which was identified in the present study.

Overall, we provide evidence that host-associated bacterial communities can be dynamic and that it is likely that their performance on the host depends on environmental conditions. In addition, we identified possible functional plasticity of amphibian skin bacteria along a gradient in the tropics where Bd epidemics have varied in intensity, though it would be premature to conclude that the observed variation in metabolite profiles, which we used to assess potential function, represent variation in function against Bd specifically. Considering the minimal variation in community structure of these bacterial communities across sites, we suggest that further characterization of the functions of these communities could be a promising area of research to elucidate how diversity influences function across environmental gradients. Lastly, we suggest that probiotic-based amphibian conservation efforts should consider the context-dependent function of probiotic candidates within the range of environmental conditions associated with sites of future reintroductions.

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Table 1. Study sites and sample sizes taken per site. All six sites have Bd present, according to data in Lips et al. [34], Woodhams et al. [36] and based on personal communication (R. Ibáñez and J. Voyles pers. comm.).

Sampling site	Coordinates	Elevation (m.a.s.l)	Sample Sizes	
			Bacterial DNA (Bd analysis)	Metabolite
Cerro Azul	09º12.056'N, 079º24.302'W	high/mid (607)	12 (10)	12
Cerro Campana National Park	08º40.527'N, 079º55.762'W	high/mid (767)	11 (0)	15
Cope (G. D. Omar Torrijos H. National Park)	08º40.186'N, 080º35.375W	high/mid (713)	11 (10)	15
Soberanía National Park	09º 04.895'N, 079º 39.516'W	lowland (150)	10 (10)	15
LowCope (Barrigón)	08º37.712'N, 080º34.995'W	lowland (291)	11 (10)	15
Sierra Llorona	9º 20.515'N, 79º 46.604'W	lowland (207)	5 (0)	0
Total			60	72

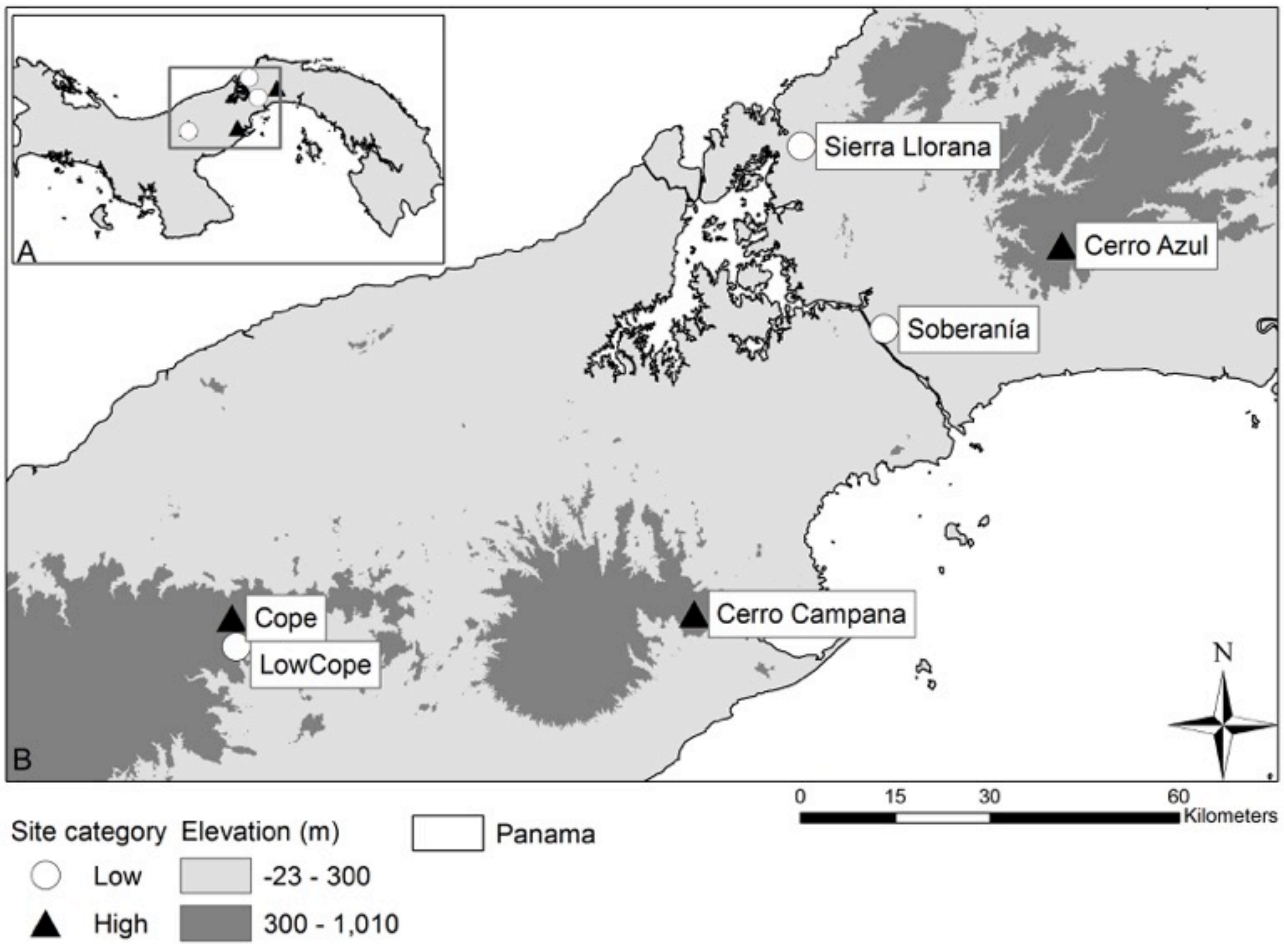
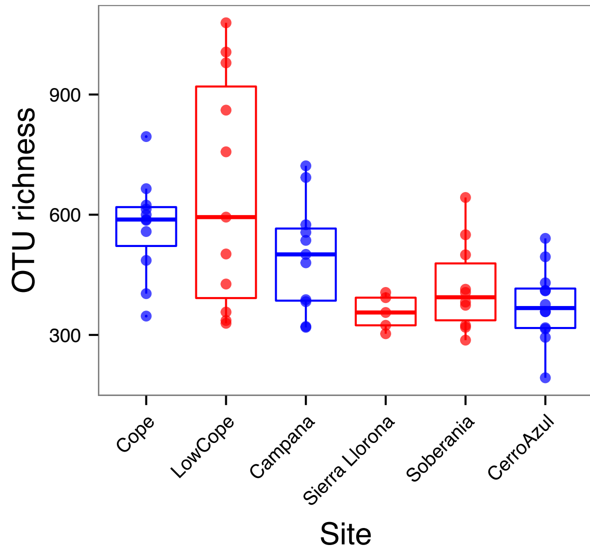
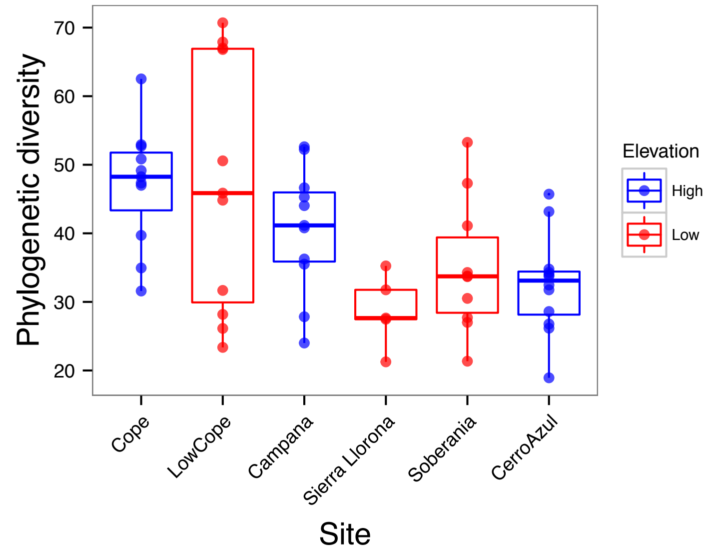


Figure 1. Map of Panamá showing the location of field sites throughout the country.





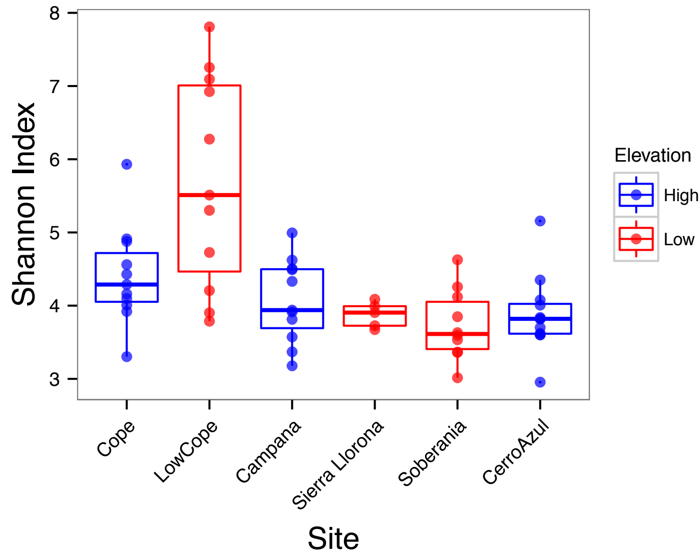
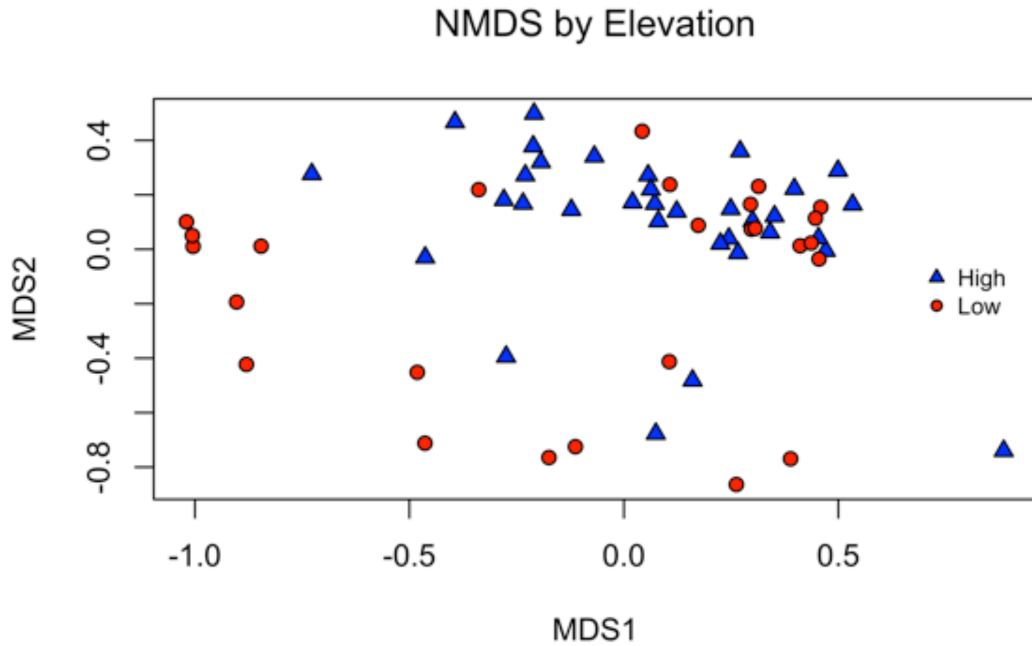


Figure 2. Alpha diversity of bacterial OTUs by site (richness, phylogenetic diversity, Shannon index).

A)



B)

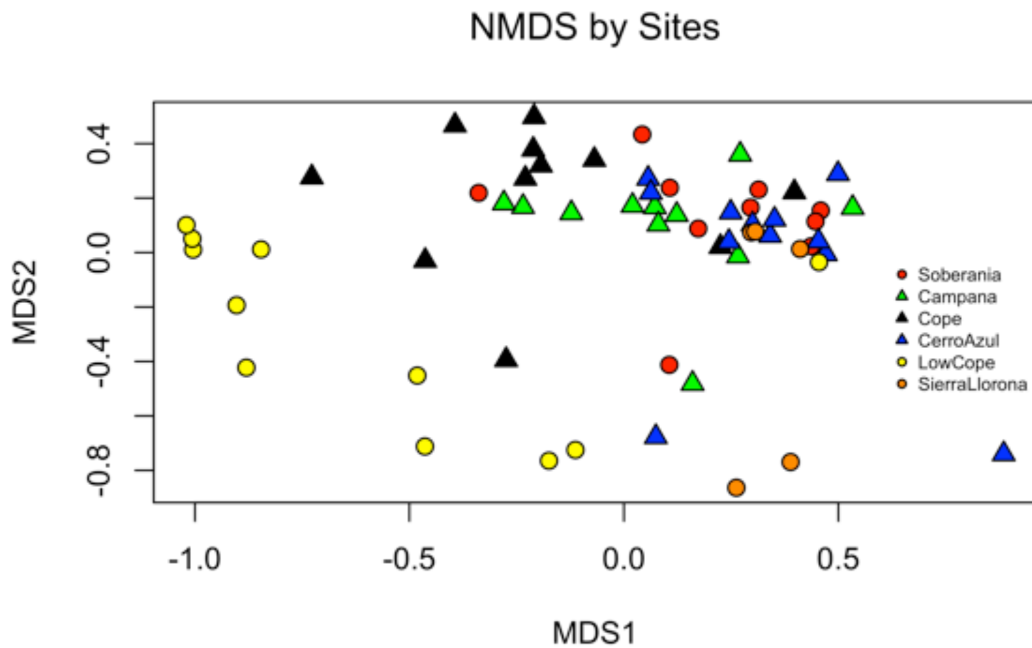


Figure 3. Beta diversity comparisons of bacterial communities using nonmetric multi-dimensional scaling (NMDS) ordinations based on Bray-Curtis dissimilarity distances between elevation categories (**a**) and among study sites (**b**). Each *poin*t represents a single frog; and points shapes: *triangles* = high elevation sites; *circles* = low elevation sites. Axis labels (MDS) represent the multi-dimensional scaling.

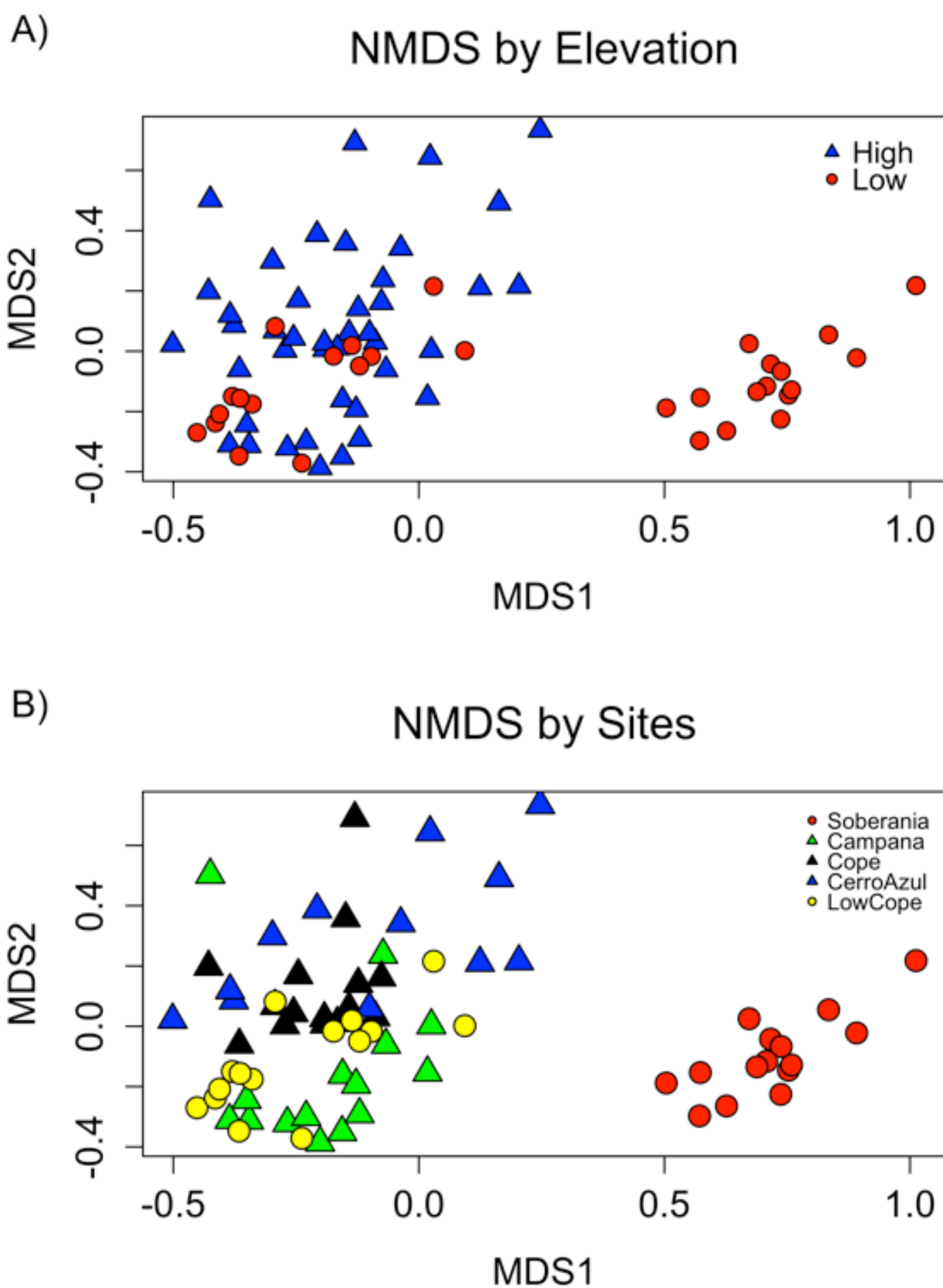


Figure 4. Beta diversity comparisons of metabolite profiles using nonmetric multi-dimensional scaling (NMDS) ordinations based on Jaccard dissimilarity distances between elevation categories (**a**) and among study sites (**b**). Each *point* represents a single frog; and points shapes: *triangles* = high elevation sites; *circles* = low elevation sites. Axis labels (MDS) represent the multi-dimensional scaling.

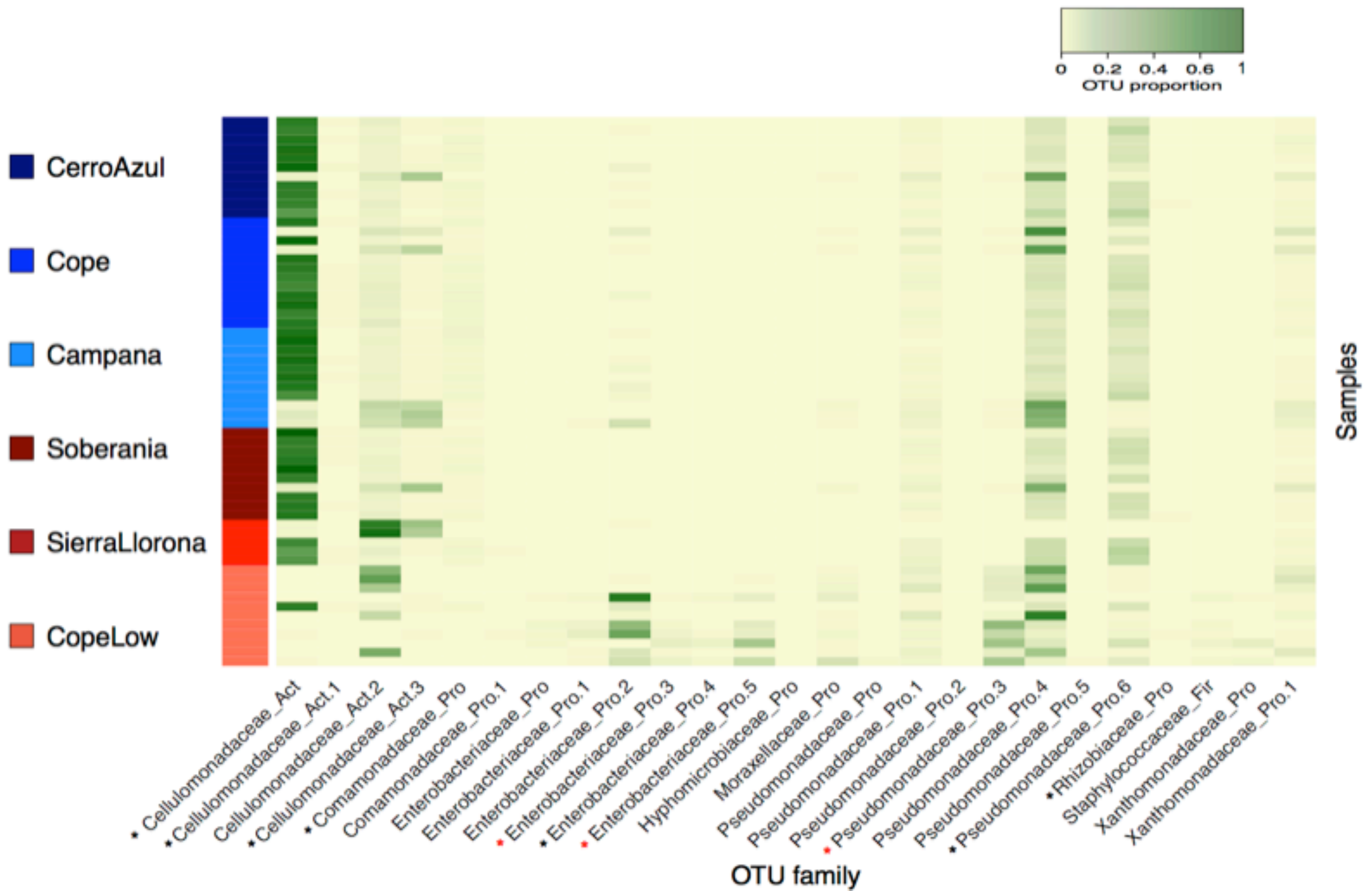


Figure 5. Heatmap of the relative abundances of the OTUs considered members of the core-microbiome across samples and sites. Each row in the heatmap represents a single frog sampled. The colors next to rows are associated with a specific site. Columns represent each core OTU. OTUs with an asterisk (*) represent OTUs with significantly different relative abundances ($p < 0.05$) across sites. Red asterisks represent OTUs with a significant higher relative abundance at the site LowCope.

Supplementary material

Table S1. Results of alpha diversity comparisons of the skin bacterial communities based on richness, Shannon Index and phylogenetic diversity. A) Comparisons between elevations and among sites comparisons from GLMM and GLM models, respectively. B) Post-hoc comparisons among sites for each diversity metric.

a)

Alpha Diversity Metric	**By Elevation**		**By Site**	
	Chisquare	*(P value)*	*F statistic*	*(P value)*
Phylogenetic diversity	0.24	0.62	5.2	**<0.001**
Shannon	0.01	0.92	10.36	**<0.001**
	Chisquare	*(P value)*	*Deviance*	*(P value)*
Richness	0.06	0.81	60.62	**<0.001**

b)

Pairwise comparisons	**Richness**		**Shannon**		**Phylogenetic Diversity**	
	z value	*P-value*	*z value*	*P-value*	*z value*	*P-value*
CerroAzul - Campana	-2.412	0.15	0.694	0.982	2.006	0.326
Cope - Campana	1.13	0.867	-1.159	0.851	-1.306	0.772
LowCope - Campana	2.325	0.181	-4.769	**<0.001**	-1.409	0.710
SierraLlorona - Campana	-2.197	0.236	0.538	0.994	2.256	0.202
Soberania - Campana	-1.383	0.734	1.188	0.838	1.279	0.787
Cope - CerroAzul	3.566	**0.005**	-1.878	0.407	-3.292	**0.012**
LowCope - CerroAzul	4.786	**<0.001**	-5.556	**<.001**	-3.391	**0.009**
SierraLlorona - CerroAzul	-0.337	0.999	0.007	1.000	0.878	0.949
Soberania - CerroAzul	0.94	0.935	0.54	0.994	-0.652	0.986
LowCope - Cope	1.195	0.837	-3.67	**0.003**	-0.104	1
SierraLlorona - Cope	-3.088	0.024	1.422	0.706	3.071	**0.024**
Soberania - Cope	-2.486	0.126	2.303	0.186	2.507	0.115
SierraLlorona - LowCope	-4.03	**<0.001**	4.003	**<0.001**	3.132	**0.02**
Soberania - LowCope	-3.651	**0.003**	5.691	**<0.001**	2.602	0.09
Soberania - SierraLlorona	1.062	0.895	0.419	0.998	-1.332	0.757

Table S2. Parameter estimates and P values from GLM models determining the effect of geographical longitude (converted into UTM) on alpha diversity estimates of the skin microbial communities. Parameter estimates are shown on a logit scale, which can be transformed as $e^x/(1+e^x)$.

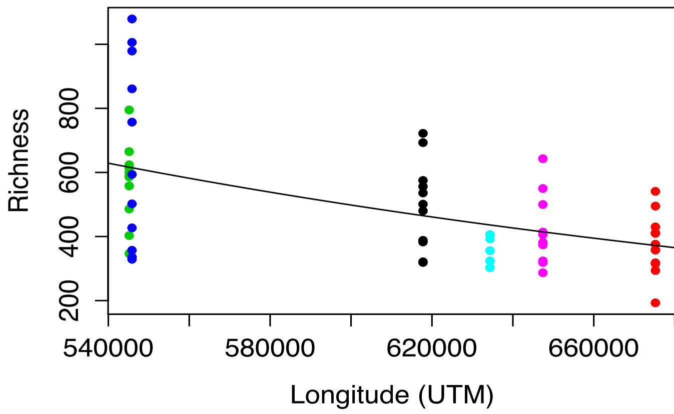
Diversity metric	Parameter	Estimate	SE	*P*
Richness	*Intercept*	8.538	0.4443	<0.001
	Longitude	-3.88E-06	7.27E-07	**<0.001**
Shannon	*Intercept*	-1.03E-01	6.40E-02	0.113
	Longitude	5.55E-07	1.07E-07	**<0.001**
Phylogenetic diversity	*Intercept*	-2.07E-02	9.91E-03	0.0411
	Longitude	7.66E-08	1.67E-08	**<0.001**

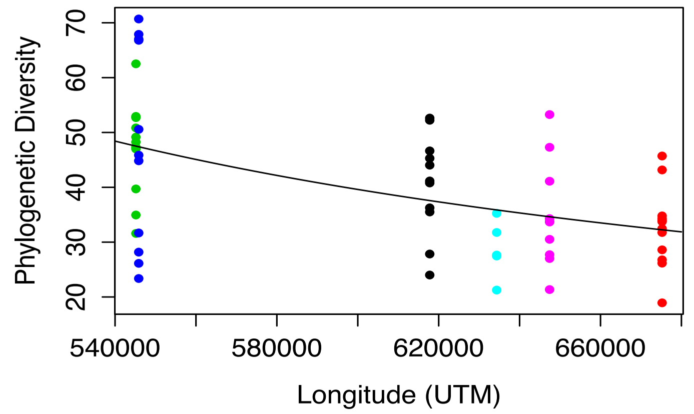
Table S3. Results of pair-wise comparisons of metabolite profiles between sites using PERMANOVA based on Jaccard dissimilarity distances. The comparison 'All Sites (Soberanía excluded)' was conducted to determine whether the site Soberanía was the driver of the significant differences among sites in the inclusive analysis given its isolation in the ordination (Fig. 4b).

Pair-wise Comparison	*F*	R^2	*P-value*
All Sites (Soberanía exluded)	4.4254	0.20032	**0.0001**
Soberanía vs. Cope	46.171	0.62249	**0.0001**
Soberanía vs. Low Cope	43.78	0.60992	**0.0001**
Soberanía vs. Campana	36.853	0.56825	**0.0001**
Soberanía vs. Cerro Azul	22.295	0.47141	**0.0001**
Cope vs. Low Cope	4.2789	0.13256	**0.0012**
Cope vs. Campana	6.0045	0.17658	**0.0003**
Cope vs. Cerro Azul	2.4114	0.08797	**0.0171**
LowCope_vs_Campana	2.0406	0.06793	0.0586
Low Cope vs. Cerro Azul	5.5085	0.18056	**0.0002**
Campana vs. Cerro Azul	6.0604	0.19512	**0.0001**

Table S4. Taxonomic information, based on the RDP classifier including the Green Genes 13_5 OTU ID number, and mean relative abundances of the 25 core OTUs based on a 90% prevalence cut-off across all 60 frogs from all 6 sites. OTUs are ordered in a decreasing number based on their mean relative abundances.

OTU ID number	Label in Figure #5	Phylum	Family	Genus	Mean relative abundance (%)
235695	Cellulomonadaceae_Act	Actinobacteria	Cellulomonadaceae	Cellulomonas	45
394796	Pseudomonadaceae_Pro.4	Proteobacteria	Pseudomonadaceae	Pseudomonas	15.5
4378239	Cellulomonadaceae_Act.2	Actinobacteria	Cellulomonadaceae	-	9
4451011	Pseudomonadaceae_Pro.6	Proteobacteria	Pseudomonadaceae	Pseudomonas	8.1
4473756	Cellulomonadaceae_Act.3	Actinobacteria	Cellulomonadaceae	Cellulomonas	3.9
924547	Enterobacteriaceae_Pro.2	Proteobacteria	Enterobacteriaceae	-	3.7
269930	Pseudomonadaceae_Pro.3	Proteobacteria	Pseudomonadaceae	-	2.5
410048	Pseudomonadaceae_Pro.1	Proteobacteria	Pseudomonadaceae	Pseudomonas	2.4
1139932	Xanthomonadaceae_Pro.1	Proteobacteria	Xanthomonadaceae	-	2.2
4456068	Comamonadaceae_Pro	Proteobacteria	Comamonadaceae	-	1.6
2119418	Enterobacteriaceae_Pro.5	Proteobacteria	Enterobacteriaceae	-	1.2
4449458	Moraxellaceae_Pro	Proteobacteria	Moraxellaceae	Acinetobacter	0.9
4350881	Cellulomonadaceae_Act.1	Actinobacteria	Cellulomonadaceae	-	0.7
252822	Rhizobiaceae_Pro	Proteobacteria	Rhizobiaceae	Agrobacterium	0.4
denovo9513	Enterobacteriaceae_Pro.1	Proteobacteria	Enterobacteriaceae	-	0.4
814442	Enterobacteriaceae_Pro.3	Proteobacteria	Enterobacteriaceae	Citrobacter	0.4
4345285	Staphylococcaceae_Fir	Firmicutes	Staphylococcaceae	Staphylococcus	0.3
denovo53436	Comamonadaceae_Pro.1	Proteobacteria	Comamonadaceae	-	0.3
91962	Enterobacteriaceae_Pro	Proteobacteria	Enterobacteriaceae	-	0.2
668514	Enterobacteriaceae_Pro.4	Proteobacteria	Enterobacteriaceae	-	0.2
denovo40863	Pseudomonadaceae_Pro.5	Proteobacteria	Pseudomonadaceae	-	0.2
1119668	Hyphomicrobiaceae_Pro	Proteobacteria	Hyphomicrobiaceae	Devosia	0.1
818602	Pseudomonadaceae_Pro	Proteobacteria	Pseudomonadaceae	Pseudomonas	0.1
denovo28229	Pseudomonadaceae_Pro.2	Proteobacteria	Pseudomonadaceae	Pseudomonas	0.1
81358	Xanthomonadaceae_Pro	Proteobacteria	Xanthomonadaceae	-	0.02





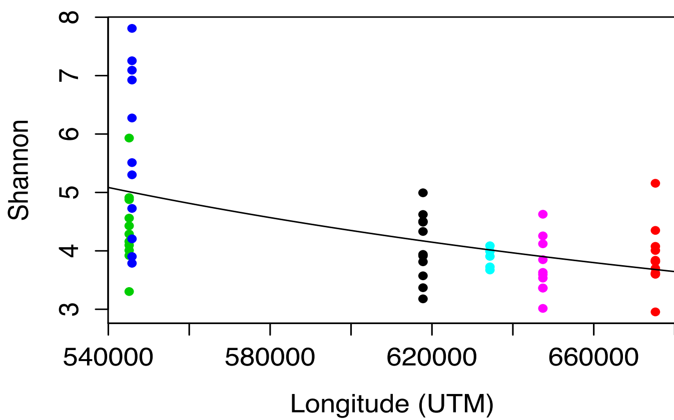


Figure S1. Graph showing a trend line between the site geographic longitude and the alpha diversity metric based on GLMMs. Parameter estimates of the GLMMs were backtransformed to the scale of the data, and each point in the figure represent a single sample (i.e. frog).

Chapter 3: Culture Media and Individual Hosts Affect the Recovery of Culturable Bacterial Diversity from Amphibian Skin

Medina D, Walke JB, Gajewski Z, Becker MH, Swartwout MC and Belden LK. 2017. Culture Media and Individual hosts Affect the Recovery of Culturable Bacterial Diversity from Amphibian Skin. *Frontiers in Microbiology* 8:1574. doi:10.3389/fmicb.2017.01574

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Abstract

One current challenge in microbial ecology is elucidating the functional roles of the large diversity of free-living and host-associated bacteria identified by culture-independent molecular methods. Importantly, the characterization of this immense bacterial diversity will likely require merging data from culture-independent approaches with work on bacterial isolates in culture. Amphibian skin bacterial communities have become a recent focus of work in host-associated microbial systems due to the potential role of these skin bacteria in host defense against the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd), which is associated with global amphibian population declines and extinctions. As there is evidence that some skin bacteria may inhibit growth of Bd and prevent infection in some cases, there is interest in using these bacteria as probiotic therapy for conservation of at-risk amphibians. In this study, we used skin swabs from American toads (*Anaxyrus americanus*) to: 1) assess the diversity and community structure of culturable amphibian skin bacteria grown on high and low nutrient culture media; 2) determine which culture media recover the highest proportion of the total skin bacterial community of individual toads relative to culture-independent data; and 3) assess whether the plated communities from the distinct media types vary in their ability to inhibit Bd growth in *in-vitro* assays. Overall, we found that culture media with low nutrient concentrations facilitated the growth of more diverse bacterial taxa and grew distinct communities relative to media with higher nutrient concentrations. Use of low nutrient media also resulted in culturing proportionally more of the bacterial diversity on individual toads relative to the overall community defined using culture-independent methods. However, while there were differences in diversity among media types, the variation among individual hosts was greater than variation among media types, suggesting that swabbing more individuals in a population is the best way to maximize culture collections, regardless of media type. Lastly, the function of the plated communities against Bd did not vary across culture media type or between high and low nutrient media. These results inform current efforts for developing a probiotic-based approach for amphibian conservation and help to ensure that culture collections are capturing the majority of the important diversity in these systems.

Introduction

Microbial ecologists currently face the challenge of characterizing the ecological role of the vast bacterial diversity associated with different environments and hosts, identified by culture-independent methods, such as 16S rRNA gene amplicon sequencing (Vartoukian et al., 2010; Zengler et al., 2002). A complete understanding of the metabolic, pathogenic and functional features of this diversity will be enhanced by the use of "-omics" methods, such as shotgun metagenomics, the cultivation and isolation of the bacterial species (Zengler et al., 2002) and by linking culture-independent and culture-dependent approaches (Rebollar et al., 2016a).

Importantly, culturable bacteria have provided a good system for assessing ecological processes (Jessup et al., 2005). For instance, bacterial systems have been used to assess the generality of some key factors known to influence and determine diversity patterns in plants and animals, such as habitat type, habitat heterogeneity, disturbance and primary productivity (reviewed in, (Horner-Devine et al., 2004). In addition, expanding our understanding about the natural history of bacteria, which are essential in critical natural processes, such as nutrient cycling, requires at some level the use of cultured isolates (Jessup et al., 2004). Lastly, the cultivation of bacteria can play a key role in the development of probiotics to mitigate pathogen infection in an array of species (Bletz et al., 2013; Walke and Belden, 2016). While "-omics" approaches can assist with identification of potential probiotics, implementation will still require the cultivation of bacteria for biological assays and probiotic application (e.g., Rebollar et al., 2016a).

Bacterial communities associated with amphibian skin have received attention due to the potential defensive role they play against the amphibian fungal skin pathogen *Batrachochytrium dendrobatidis* (Bd) (Bletz et al., 2013; Walke and Belden, 2016). The potential defensive role of the amphibian skin bacteria is important from an ecological and conservation standpoint given that chytridiomycosis, the disease caused by Bd, has been associated with amphibian extinctions and population declines around the world (Berger et al., 1998; Bosch and Martínez-Solano, 2006; Crawford et al., 2010; Lips et al., 2006; Vredenburg et al., 2010), and has caused a substantial disease-related loss of biodiversity (Fisher et al., 2012; Lips, 2016). Amphibian skin bacterial communities might also serve as a microbial system to address fundamental ecological questions, in particular those focused on the processes influencing diversity, community assembly, and function. For example, amphibian skin bacterial communities have been used to determine the roles of neutral (e.g. dispersal and ecological drift) and deterministic processes (e.g. habitat filtering and competition) in shaping bacterial communities (Loudon et al., 2016), to assess the influence and feedbacks caused by an invasive species, such as a host skin pathogen (Jani and Briggs, 2014), to examine the relationship between community structure and function (Becker et al., 2015; Belden et al., 2015; Walke et al., 2015b), and to elucidate the factors potentially driving context-dependent function (Daskin et al., 2014; Loudon et al., 2014; Woodhams et al., 2014).

The use of culture-dependent techniques has played an important role in the study of bacteria associated with amphibian skin, mainly within a conservation context to assist in the development of a probiotic-based conservation method. Culture-dependent techniques have facilitated the development of protocols to identify skin bacterial isolates that inhibit Bd growth *in vitro* (e.g. Becker et al., 2015; Bell et al., 2013; Flechas et al., 2012; Harris et al., 2006; Lauer et al., 2007; 2008), and also to identify some of the ecological factors (e.g. interspecific competition and temperature) affecting the production of bacterially-produced secondary metabolites (Daskin et al., 2014; Loudon et al., 2014).

In ecology, studies manipulating nutrient concentration and composition have advanced understanding of the relationship between primary productivity and diversity in terrestrial plant communities (Goldberg and Miller, 1990; Wilson and Tilman, 1991), aquatic communities (Schindler, 1990) and microbial systems (Kassen et al., 2000). Within this context, when cultivating bacteria, the composition and diversity of the cultured community is limited by factors such as incubation time and culture media nutrient concentration and composition (Stevenson et al., 2004). Thus, nutrient composition and availability can influence bacterial communities and enhance the cultivation of previously uncultured bacteria (Vartoukian et al., 2010). For instance, nutrient-rich culture media (i.e. complex media) favors the growth of fast-

growing bacteria over slower-growing bacteria (Connon and Giovannoni, 2002; Vartoukian et al., 2010), and the dilution of nutrients in culture media has been used to cultivate previously uncultured bacteria (Connon and Giovannoni, 2002; Vartoukian et al., 2010; Zengler et al., 2002).

In this study, we aimed, from an ecological perspective, to assess how high and low nutrient culture media influence the diversity of cultured amphibian skin bacteria that are recovered. In addition, and within an applied context, we also assessed the ability of different culture media types to grow a high portion (i.e. number of OTUs) of amphibian skin bacteria, and determined what culture media can recover the most representative fraction of the bacterial community relative to a culture-independent method. Lastly, given the ability of some bacterial isolates to inhibit Bd growth *in vitro*, particularly when grown with other bacteria (Loudon et al., 2014), and their potential as a conservation approach to mitigate Bd infection, we aimed to determine whether the bacterial communities growing on the different media types differed in their ability to inhibit Bd growth.

Materials and Methods

Sample Collection

We analyzed bacterial communities from skin swabs from 12 American toads (*Anaxyrus americanus)* collected in Jefferson National Forest near Blacksburg, VA (USA). In the field, toad skin bacterial communities were sampled as described by Walke et al. (2015a). Briefly, each individual toad was handled with a new pair of nitrile gloves, rinsed twice with sterile water to remove transient microbes (Belden et al., 2015; Walke et al., 2014), and swabbed sequentially with two sterile rayon swabs (MW113; Medical Wire & Equipment). The swabbing technique was standardized and consisted of 20 strokes on the ventral side of the toad and 5 strokes along each thigh and foot. The first swab was placed in a 1.5 ml sterile microcentrifuge tube and was used to characterize the skin bacterial communities via culture-independent 16S rRNA gene amplicon sequencing. The second swab was stored in another 1.5ml sterile microcentrifuge tube containing 100 µl of TSYE-glycerol medium (2% Trypticase soy broth, 1% yeast extract, 20% glycerol) and was used for the characterization of bacterial isolate communities grown on four different types of culture media. These cultured communities were also subsequently characterized via 16S rRNA gene amplicon sequencing and were tested for whole community *in vitro* inhibition of Bd growth. Both field-collected swabs were stored at -80C until processing. All animal use was approved by the Institutional Animal Care and Use Committees of Virginia Tech.

Culture-Independent Assessment of the Skin Bacterial Communities

DNA was extracted from the first swab using the Qiagen DNeasy blood and tissue kit (Valencia, CA) protocol for Gram positive bacteria, with an initial incubation step of 1 h at 37ºC. For the community characterization, the V4 region of the 16S rRNA gene was amplified using the primers 515F and the barcoded 806R and sequenced using a 250 bp paired-end strategy on the Illumina MiSeq platform as described by Walke et al. (2015a). The culture-independent data produced and used in the present study was part of Walke et al. (2015a), and is available in NCBI's Sequence Read Archive (SRA) under the accession number SRP062395.

Bacterial Community Comparison among Culture Media

We plated the second swab onto four different media types to compare the bacterial isolate richness (alpha-diversity) and community structure (beta-diversity). The culture media types comprised two with a high concentration of nutrients, LB (Luria-Bertani, Fisher Scientific) and TSA (Tryptic soy agar, Remel), and two with a low concentration of nutrients, R2A (Reasoner's 2A, Difco, Becton, Dickinson and Company) and dR2A (1/10 dilution of R2A with an addition of granulated agar so the amount of agar was the same as in the undiluted R2A). We chose commonly used media for our study. For example, LB is often used to culture and maintain *Escherichia coli*, TSA is often used as a non-selective media for general purposes, and R2A, which was initially developed to culture bacteria from potable water (Reasoner and Geldreich, 1985), is the most commonly used media for culturing bacteria from amphibian skin (Antwis et al., 2015; Flechas et al., 2012; Harris et al., 2006; Walke et al., 2015a). For this study, we tested each of the four media types with an aliquot of the TSYE-glycerol solution from each of the 12 toads, resulting in 48 culture plates. We inoculated 30 µl of a briefly vortexed 1:10 dilution of the TSYE-glycerol solutions from the freezer stock of the culture swab onto each media type, followed by spreading the solution across the plate. In addition, a non-inoculated plate for each media type was used as a control to assess the potential for contamination of the media. Following inoculation, all plates were incubated at room temperature in the lab (~24 ºC).

Collection of Bacterial Cultures from Plates

After 6 days of incubation, when bacterial colonies started to cover the plates in the high nutrient concentration media, we sampled the entire bacterial community on each plate by applying a slightly modified version of the plate wash PCR procedure (PWPCR) developed by Stevenson et al. (2004). Culture plates were flooded with 3 ml of 1% tryptone broth, and a sterile spreader was used to suspend all visible bacterial colonies. Then the tryptone broth with the suspended bacteria was collected and transferred to a 2 ml sterile collecting tube and centrifuged at 10,000 rpm for 5 minutes. The resulting supernatant potentially containing bacterially-produced secondary metabolites was filtered through a 0.22 µm filter, and the cell-free supernatant (CFS) was used to conduct *in vitro* challenge assays to determine whether there was community-level variation in the ability to inhibit Bd growth among media types. After removing the supernatant for the challenge assays, we added 2 ml of MicroBead solution (MoBio Laboratories, from Ultraclean Microbial Isolate kit) to each tube of pelleted bacteria and then vortexed to homogenize the solution. These were stored at -20ºC prior to DNA extraction. DNA from these cultured bacterial communities was extracted by adding 50 µl of lysozyme solution (20 mg/ml) to each tube followed by an incubation at 56ºC for 45 min (Stevenson et al., 2004). After incubation, DNA extractions were performed following the manufacturer's protocol of the UltraClean microbial DNA isolation kit (MoBio Laboratories), which yielded a volume of 100 µl of template DNA.

Characterization of the Cultured Bacterial Communities

Following DNA extraction, assessment of the cultured communities was done as for the initial culture-independent swab, following methods of Walke et al. (2015a), with the exception that we used 2 µl of DNA template in the PCR. Out of the 48 experimental samples, five (two from the TSA culture media and one from each of the other culture media) were removed from the dataset because they would not amplify. In addition, out of the four control plates, only two could be included because most contained too little DNA for sequencing (as was expected), and

even the two that were included had very little DNA. From each of the 45 remaining samples, 200 ng of PCR product was pooled to make a composite sample, which was then cleaned with the QIAquick PCR Purification Kit (Qiagen, Valencia, California). The final pooled sample was sent for sequencing on an Illumina Mi-Seq instrument at the Dana-Farber Cancer Institute of Harvard University following Caporaso et al. (2012) using a 250 bp single-end strategy.

Raw forward 16S rRNA amplicon sequences were demultiplexed and quality-filtered using the default parameters of the Quantitative Insight into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010b), with a few exceptions: we allowed for no errors in the barcodes, increased the number of minimum consecutive low-quality base calls allowed before truncating a read (r) to 10, and decreased the fraction of the minimum number of consecutive high-quality base calls to include a read (p) to 0.5. Sequences matching PhiX, added to increase base diversity in Illumina sequencing runs, were removed from the dataset using Geneious (Biomatters, Ltd, version 8.1.8). For remaining sequences, a 97% similarity threshold was used to cluster sequences into operational taxonomic units (OTUs, ~bacterial species) using the UCLUST method (Edgar, 2010). Each OTU was represented by the most abundant sequence clustered within it, which was aligned to the Greengenes 13_8 reference database (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2010a) and assigned taxonomy using the RDP classifier (Wang et al., 2007). OTUs assigned to chloroplast or mitochondria were removed, and then OTUs with fewer than 0.01% (524 sequences) of the total reads were removed (Bokulich et al., 2012; Hughey et al., 2016). The sequencing depth per sample ranged from 3,991-164,519, including the two controls, which, as expected, had the lowest read counts at 3,991 and 8,299 reads. We removed the controls from the set of samples, given that their removal did not change the total number of OTUs, but would have reduced substantially the cut-off for the standardization of the sampling effort across samples. Thus, we rarefied the sample set to a depth of 59,000 sequences/sample. This final dataset for the culture plates consisted of 347 OTUs across the 43 samples, with 93-277 OTUs per sample (mean ± SD = 174 ± 44). 16S rRNA amplicon sequences are deposited in the NCBI's Sequence Read Archive (SRA) under the accession number SRP112779.

Comparison of Culture-Dependent and Culture-Independent Communities

To determine whether there was variation among media types in the proportion of OTUs obtained from culture plate washes relative to the culture-independent swab from the same individual, we produced a second dataset that incorporated both the amplicon data from the plate washes (N = 43) and the culture-independent swabs (N = 12). The resulting file was processed in QIIME, as described above, to produce a final OTU table containing all 55 samples. The OTU table was rarefied to 18,000 sequences/sample, which resulted in 459 total OTUs, with 54-254 OTUs per sample (mean ± SD = 148 ± 47). To avoid overestimation in analyses, 139 OTUs associated only with the culture plate samples were removed from the OTU table. These 139 OTUs occurred at very low relative abundances in the culture-independent dataset, and thus were eliminated when filtering out the OTUs below a relative abundance threshold of 0.01 and/or when rarifying the OTU table. By eliminating these OTUs, the dataset contained OTUs present only in the culture-independent swabs or shared between the culture-independent swabs and the culture plates. The final OTU table had a total of 320 OTUs, with 37-220 OTUs per sample (mean ± SD = 106 ± 43).

Community-Level Inhibition of Bd Growth across Individuals and Media Types

We also assessed whether there was variation among the cultured communities in their ability to inhibit Bd growth. We conducted *in vitro* challenge assays following the method of Bell et al. (2013) and Becker et al. (2015), with the exception that we did not initially co-culture the bacterial isolates with Bd prior to the assay because we were testing whole bacterial communities collected via plate washes. Instead, we used the cell-free supernatant (CFS) that was collected during the plate washes, as described above.

Prior to the challenge assay, zoospores from a Bd culture (JEL404, Maine-USA) were inoculated onto a 1% tryptone agar plate and grown for three days at 23ºC. The plate was then flooded with 3 ml 1% tryptone broth and the zoospore suspension collected and filtered through a 20 µm filter. The challenge assay was prepared by adding 100 µl of the CFS containing the metabolites from the bacterial communities ($N = 48$ samples; 12 samples/media type) and 100 µl of the Bd zoospore suspension (2×10^6 zoospores per ml) in each well of a sterile 96-well plate. In addition, positive and negative controls were included in each of the 96-well plates. Positive controls were prepared by adding 100 µl of the zoospore suspension and 100 µl of 1% tryptone broth. Negative controls were prepared by adding 100 µl of heat-killed zoospore suspension and 100 µl of 1% tryptone broth. Samples and controls were run in triplicate, and plates were incubated at 23ºC. Challenge assay plates were loaded the same day and with the same Bd stock solution. The optical density of each well was measured with a spectrophotometer at 492 nm immediately after plate set up was completed, and then at days 4, 7 and 11. The measurements of optical density were transformed using the formula Ln[OD/(1-OD)]. For each culture plate, the growth rate of Bd in the presence of CFS was calculated by performing a linear regression of the transformed measurements of optical density through time (day 0, 4, 7 and 11). Bd inhibition was calculated by dividing the slope of the triplicates by the average growth rate of the positive control from the respective 96-well plate, and subtracting from 1. Lastly, inhibition values of each triplicate were averaged to calculate the mean inhibition of each culture plate. Negative inhibition values suggest facilitation of Bd growth, while positive values suggest inhibition, with estimates = 1 representing a complete inhibition of Bd growth (Becker et al., 2015).

Data Analysis

Our specific goals were to: (1) compare the diversity of the cultured bacterial communities associated with the different media types representing high and low nutrient concentrations, and identify the cultured bacterial taxa that differed among the media types; (2) determine which media type cultured the highest proportion of OTUs from the full community based on the culture-independent samples; and (3) determine whether the communities growing on the different media types, or from different individual toads, differed in their ability to inhibit Bd growth. Unless noted, all statistical analyses were completed in R version 3.2.4 (R Core Team, 2016). For all generalized linear models (GLMs) and generalized linear mixed models (GLMMs), we performed visual assessments of residual plots with model predictions to confirm that the error distributions used were appropriate.

Alpha and Beta Diversity Analysis

Alpha diversity estimates were calculated with QIIME for the metrics: richness (OTUs/culture plate), Faith's phylogenetic diversity (measure of diversity based on the branch length of the phylogenetic tree) and the Shannon Index (H', which assesses community evenness). We fitted the diversity metrics to GLMMs. We considered the predictor variable

'Media' as a fixed factor and 'Individual toad' as a random factor given the nestedness of the media types within individuals. The GLMMs were performed using appropriate error distributions for the diversity metrics to account for heteroscedasticity. For richness, a negative binomial error distribution was applied to the model using the log link function. For phylogenetic diversity and the Shannon Index, which was transformed to Hill number (effective number of species; MacArthur, 1965), we used a Gamma error distribution with the inverse link function. The models were run using the R functions glmer.nb for richness, and glmer for phylogenetic diversity and the Shannon Index, from the package *lmer4* (Bates et al., 2014). Multiple comparisons were conducted with Tukey tests using the function glht in the package *multcomp* (Hothorn et al., 2008), which includes multiple comparisons for generalized linear models. Although not an explicit goal of this study, we also compared the alpha diversity estimates among individual toads since variation in total bacterial diversity across individuals can influence the culturable diversity. For instance, individual toads with high bacterial diversity had higher alpha diversity estimates in their plated communities relative to that from other individuals (Walke et al., 2015a). We used GLMs for this purpose. GLMs were performed as described above, with a negative binomial error distribution for richness estimates and a Gamma error distribution for Shannon and phylogenetic diversity.

Changes in the structure of the bacterial communities across media types were determined by the calculation of dissimilarity distances based on the Bray-Curtis distance measure, which takes into account OTU relative abundance in each community. We are only including the results based on Bray-Curtis since the Jaccard distance measure, which takes into account only the presence/absence composition of the communities, produced consistent results. A statistical comparison of the communities across media types based on the Bray-Curtis dissimilarities was done with a permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001). In addition, we compared the bacterial communities of the media types based on nutrient level, where LB and TSA were grouped as high nutrient concentration media types, and R2A and dR2A as low. When performing the PERMANOVAs, the argument "strata" was used to define the group within which to limit the permutations (i.e. individual toad) due to the nestedness of the replicates of the media types and the potential variation in the skin bacterial communities among toads. Importantly, an analysis excluding those toads that did not have replicates of all media types (toads 4, 11 and 12) was consistent with results from the inclusive analysis that is presented. Lastly, due to potential influence of the individual toads on the plated bacterial communities, as shown in the ordination (Figure 2), we also compared the communities across toads where the argument "strata" was not used when performing the PERMANOVAs. Bray-Curtis dissimilarity distances were calculated with the function vegdist, and PERMANOVAs were performed with the function adonis, both functions from the *vegan* package (Okansen et al., 2016). To visualize the results, we used principal coordinate analysis (PCoA).

Reduced variation in community structure across samples could occur on high nutrient media due to fast-growing bacteria out-competing slow-growing bacteria, and among samples from the same individual toad. As a way to test this, we compared the multivariate homogeneity of dispersion (i.e. distance from objects to cluster centroid) across media types based on Bray-Curtis dissimilarity distances. We used the function betadisper from the *vegan* package and conducted a hypothesis test to determine whether there are statistical differences in dispersion among groups using the function anova from the default R package *stats*.

Species Indicator Analysis

To identify OTUs associated with particular media types (e.g., TSA vs. LB or low nutrient concentration media vs. high nutrient concentration media), we performed an indicator species analysis using the function multipatt from the *indicspecies* package (De Cáceres and Legendre, 2009). Overall, the function multipatt quantifies, via the estimation of an Indicator Value (IndVal), the association between species (e.g., OTUs) and a group of samples (e.g., replicates within media types) based on the relative abundance and relative frequency of each species, and calculates the statistical significance of the relationships using a permutational approach (De Cáceres and Legendre, 2009).

Estimating the Proportion of OTUs Cultured Relative to the Culture-Independent Swabs

To account for the individual-level variation in OTU richness, the proportion of OTUs recovered by each media type was calculated at the individual toad level. We then used a GLMM to determine whether media types differed in the number of OTUs cultured relative to the culture-independent swabs. Similar to the alpha diversity analyses, we considered the predictor variable 'Media type' as a fixed factor and 'Individual toad' as a random factor given the nestedness of the media types within individuals. We used the Gamma error distribution with the inverse link function using the function glmer from the package *lmer4*.

Comparing Community Level Ability to Inhibit Bd Growth among Culture Media Types and Individual Toads

We compared the functional ability to inhibit Bd growth of each bacterial community growing on the culture media plates. We fitted the mean inhibition values to a linear mixed effects model given that the data were normally distributed, as determined by the Lilliefors normality test (function lillie.test from the package *nortest*; Gross and Ligges 2015). The model included the predictor variable 'Media' as a fixed factor and 'Individual toad' as a random factor. In addition, to assess the potential effect of individual toads in the inhibition values, we fitted the mean inhibition values to a linear model using the variable 'toad' as the predictor variable. The linear mixed effect model was performed using the function lmer from the package *lmer4* (Bates et al., 2014), and the linear model was performed using the function lm from the default R package *stats*.

Results

Alpha diversity metrics differed significantly among media types for richness and Faith's phylogenetic diversity (richness: Chisq = 15.68, $P = 0.0013$; phylogenetic diversity: Chisq = 25.67, $P < 0.0001$), with the low nutrient concentration media, R2A and dR2A, having a significantly higher diversity compared to the high nutrient concentration media, LB and TSA (Figures 1A, B, Richness: LB-dR2A: $z = -3.23$, $P = 0.007$; TSA-dR2A: $z = -3.52$, $P = 0.002$; R2A-LB: $z = 2.91$, $P = 0.019$; R2A-TSA: $z = -3.23$, $P = 0.007$; Faith's phylogenetic diversity: LB-dR2A: $z = 4.82$, $P < 0.001$; TSA-dR2A: $z = 3.84$, $P < 0.001$; R2A-LB: $z = -4.58$, $P < 0.001$; R2A-TSA: $z = 3.61$, $P = 0.002$). There were no significant differences in the pairwise comparisons between media types within nutrient concentration levels. In contrast to richness and Faith's phylogenetic diversity, we did not find significant differences when comparing the diversity among media types based on Shannon Index Hill numbers (Shannon Hill number: Chisq = 7.17, P = 0.067), although there was a trend for dR2A and R2A to be higher (Figure 1C). There were also significant differences in the alpha diversity estimates of the plated

communities among toads (richness: Deviance = 42.59, $P < 0.001$; phylogenetic diversity: *F statistic* = 3.96, $P = 0.001$; Shannon Hill number: *F statistic* = 6.68, $P < 0.001$).

Bacterial community structure differed among the four media types and also between high and low nutrient concentration media (Figure 2, media types: pseudo-F = 0.68, $R^2 = 0.05$, $P = 0.002$; nutrient concentration: pseudo-F = 1.14, $R^2 = 0.03$, $P < 0.001$). However, differences among individual toads explained substantially more variation (pseudo-F = 5.32, $R^2 = 0.65$, $P < 0.001$). Lastly, the average distances to centroids (~multivariate variance) did not differ significantly among the media types ($F = 0.83$, $P = 0.48$; dR2A 0.57; R2A 0.60; LB 0.61; TSA 0.61). In contrast, distances to centroids did differ among individual toads (Figure 3, $F = 14.57$, $P = 0.001$), which supports the substantial variation explained by individual toads mentioned above, and suggests that individual toads are the main drivers of the observed clustering in Figure 2.

We identified 50 indicator OTUs that were significantly ($p < 0.05$) associated with either a specific culture media or high/low nutrient media types. The distribution of these indicator OTUs across media types was: six associated with dR2A, one with TSA, and 41 with the low nutrient media group of R2A and dR2A, suggesting that the low nutrient agars did pick up a unique set of bacteria (Table 1, Figure 4, Figure 5). In addition, there were two indicator OTUs absent in only one of the 4 media types (Table 1): one absent only in LB and another one in dR2A. The relative abundance distribution of the indicator OTUs suggested that some taxa seemed to favor a particular type of culture media. For example, the family *Pseudomonadaceae* had higher relative abundance on LB media, the family *Phyllobacteriaceae* on TSA media, and the families *Sphingomonadaceae* and *Xanthomonadaceae* on the low nutrient media group, R2A and dR2A (Figure 5). Lastly, we also found that a higher proportion of OTUs from the culture-independent swabs were cultured on R2A and dR2A, relative to LB and TSA media plates (Chisq = 26.19, $P < 0.0001$; mean ± sd: R2A 59.65% ± 13.88; dR2A 60.71% ± 14.29; LB 48.47% ± 9.5; TSA 45.38% ± 14.31). This result is further supported by the lower Bray-Curtis dissimilarity distances between the cultured communities and those from the culture-independent swabs (Figure 6).

Despite differences across media types in community structure and proportion of recovered OTUs from the culture-independent swabs, we did not find differences among media types in the ability of metabolites from the plated communities to inhibit Bd growth (Figure 7, Chisq = 4.14, $P = 0.25$; mean ± sd: R2A 0.15 ± 0.53; dR2A 0.15 ± 0.25; LB 0.36 ± 0.52; TSA 0.44 ± 0.53). Likewise, despite differences among toads in community structure, we also did not find significant differences in the inhibition values among them ($F = 1.55$, $P = 0.16$). Overall, inhibition estimates across samples ranged from -1.16 (facilitating Bd growth) to 1.01 (completely inhibiting Bd growth), which suggests a substantial amount of variation across samples.

Discussion

We used amphibian skin bacterial samples to conduct a comparative analysis of the diversity and community structure among plated communities growing on media that varied in nutrient concentration. We addressed the present study from two perspectives: first, from an ecological perspective that aimed to assess how variation in productivity (i.e. distinct nutrient concentrations in the culture media) influences the diversity of the cultured bacterial communities. Second, from an applied perspective that aimed to determine what culture media recovers the highest fraction of the amphibian skin bacterial communities relative to data derived

from a culture-independent approach, and to assess whether the plated communities differ in their ability to inhibit the growth of an amphibian pathogenic fungus (Bd strain JEL404). Overall, we found that culture media with low nutrient concentration, R2A and dR2A, facilitated the growth of more diverse and distinct communities relative to the culture media with higher nutrient concentration, LB and TSA. In addition, the high bacterial diversity observed and exclusively associated with R2A and dR2A reflects a higher proportion of the culturable diversity that can be recovered from amphibian skin. However, the estimated function of the plated communities against Bd, based on the whole-plate wash method, did not vary across the culture media or productivity levels. Interestingly, we found that variation among individual hosts influenced the structure of the plated bacterial communities more than the media type. Thus, when attempting to maximize diversity of cultured isolates, swabbing more individuals in the population may be more valuable than using diverse media (Walke et al., 2015a).

The relationship between primary productivity and diversity is considered a key factor determining spatial and temporal diversity patterns in ecological systems (Jessup et al., 2004). Interestingly, in contrast to the general positive quadratic pattern observed in plants and animals (Rosenzweig 1995), diversity patterns across productivity gradients in microbial systems have been inconsistent across studies (Horner-Devine et al., 2004). The relationship between productivity and diversity in microbial systems has been examined via correlational studies focusing on the variation of bacterial diversity across gradients of nutrient concentration in both laboratory and field studies (Benlloch et al., 1995; Bohannan and Lenski, 2000; Kassen et al., 2000; Horner-Devine et al., 2003). Though our study did not examine the effects of a concentration gradient of a specific nutrient, it did represent microcosms with distinct arrays of nutrients at different concentrations. Moreover, the observed results are consistent with a study showing that diversity patterns in microbial systems can be mediated by the role of ecological factors, such as changes in the relative importance of competition across a gradient of nutrient concentrations (Bohannan and Lenski, 2000). For instance, the observed higher alpha diversity estimates in culture media with low concentrations of nutrients, relative to nutrient-rich ones, highlight the role of competition in plated communities, where faster-growing bacteria tend to outcompete slow-growing bacteria. In addition, culture media with high concentrations of nutrients favoring fast growing bacteria can limit the occurrence of positive relationships between bacterial species, such as cross-feeding and co-aggregation (Vartoukian et al., 2010; Faust and Raes, 2012), which might enhance the growth of slow-growing bacterial taxa.

We observed variation in the structure of the plated communities across media types even though most of the observed variation was driven by differences among individual toads. The observed variation across media types can be explained, in part, due to the fact that culture media types are commonly developed for different purposes and with different degrees of selectivity. For instance, LB and TSA are considered non-selective complex media (i.e. nutrient-rich), whose composition is poorly defined because they usually include complex ingredients, such as yeast extract (Slonczewski and Foster 2017). In contrast, R2A is considered a defined medium whose chemical components are known (Slonczewski and Foster 2017) and which selects for slow-growing bacteria, in particular those from aquatic environments (Reasoner and Geldreich, 1985). In the present study, the number and taxonomic classification of the indicator OTUs associated with R2A and dR2A suggests that R2A is the most appropriate media to recover a high diversity of the culturable members of the amphibian skin community. In addition, R2A also recovers some of the most abundant and prevalent taxa occurring on amphibian skin, based on studies of tropical and temperate species (Becker et al., 2015; Belden et al., 2015; Kueneman et al., 2013;

Medina et al., 2017; Rebollar et al., 2016b; Walke et al., 2014). For example, we identified several indicator OTUs from the families *Nocardiaceae*, *Enterobacteriaceae*, *Pseudomonadaceae*, *Sphingomonadaceae* and *Xanthomonadaceae* associated with R2A and dR2A that were the most abundant and prevalent bacterial taxa on these individual toads according to 16S rRNA amplicon data (Walke et al., 2015a). Thus, the selection for slow-growing bacteria that characterizes R2A could facilitate the recovery of a higher diversity of culturable bacteria from these communities relative to LB and TSA, which are non-selective complex media and allow faster-growing bacteria to outcompete slow-growing bacteria.

The study by Walke et al. (2015a) that used the same toads aimed to examine the cultured portion of the amphibian skin bacterial communities identified with a culture-independent method, and determined that for each individual toad an average of 0.95% of the community was recovered using R2A. The recovery value estimated by Walke et al. (2015a) is substantially lower compared to that estimated in our study using R2A (i.e. 60%). There are a number of differences in the methods between our study and Walke et al. (2015a) that likely contribute to the observed differences in the number of OTUs recovered. For example, the sampling technique (plate wash in our study vs. pure culture isolation in Walke et al., 2015a) likely influenced the final set of cultured isolates. Pure culture isolation requires manually selecting morphologically-distinct isolates in serial plating, which could result in over-looking related taxa that are morphologically similar, or missing those that require longer incubation periods to have visible colony formation. This could result in an under-estimation of what is cultured. In addition, our present study used a filtering cutoff of 0.01% (maintained only those OTUs with greater than 0.01% relative abundance), whereas Walke et al. (2015a) used a cutoff of 0.001%. The higher cutoff value 0.01% reduces the number of OTUs in the dataset, which could result in a higher overlap among cultured and culture-independent samples in terms of the OTU composition. We think a cutoff of 0.01%, which was determined in the present study as the cutoff level at which OTU richness leveled off in the dataset (per Bokulich et al., 2012), potentially represents a more realistic view of the actual bacterial species present in the community (Bokulich et al., 2012; Hughey et al., 2016).

Bacterial secondary metabolites produced in response to interspecific interactions have been suggested as a mechanism by which skin bacterial communities can protect their amphibian hosts against Bd infection (Bletz et al., 2013; Walke and Belden, 2016). Furthermore, recent evidence suggests that co-culturing bacterial isolates from amphibian skin can enhance the production of emergent antifungal metabolites, which then have a stronger inhibitory ability against Bd growth relative to monocultures (Loudon et al., 2014; Piovia-Scott et al., 2017). Within the context of our study, even though we observed variation in the diversity of the plated communities across culture media and individual toads, we found no significant differences across these factors in the ability to inhibit Bd growth in challenge assays. In fact, we found a substantial amount of variation, ranging from plated communities that completely inhibited Bd growth to others that enhanced growth. This result is somewhat surprising considering that the cell-free supernatant collected from each of the plated communities should have comprised bacterially-produced secondary metabolites resulting from interspecific interactions. However, given the high diversity of taxa that grew on the plates, variation in density of the distinct bacterial taxa across plated communities, combined with stochastic variation, could have influenced our results (Loudon et al., 2014). Moreover, it is possible that we could have induced additional variation with the method used for collecting the bacterial community and the supernatant from each culture plate (i.e. plate wash; Stevenson et al., 2004). To our knowledge,

no previous studies have used the plate wash approach to collect bacterially-produced metabolites. We based this approach on the plate wash method of Stevenson et al. (2004) that was used to survey bacterial communities, as we were interested in trying to get a whole community estimate of potential Bd inhibition. This method was applied under the assumption that anti-fungal metabolites might be produced, at least in part, on the surface of the solid media because the zone of Bd inhibition is commonly observed on the surface of culture plates during Bd challenge assays (Flechas et al., 2012; Harris et al., 2006; Woodhams et al., 2007). Further research will likely be required to fine tune and verify this method as a viable option for assessing whole community Bd inhibition. Our method also did not include the step of co-culturing the plated bacterial communities with Bd, as has been done in previous studies (Becker et al., 2015). However, Becker et al. (2015) found that co-culturing bacterial isolates with Bd had no effect on the ability of bacterial isolates to inhibit Bd growth.

Using culture media with low nutrient concentrations and/or diluted culture media is known to improve the probability of capturing a better representation of bacterial communities living in nature (Vartoukian et al., 2010). The present study provides evidence that low nutrient R2A, relative to other common culture media, allows the growth of a higher diversity of bacterial taxa and recovers a higher proportion of the overall diversity occurring on the amphibian skin. These findings are relevant given that R2A has been widely used in the isolation of amphibian skin bacteria (e.g. Antwis et al., 2015; Becker et al., 2015; Daskin et al., 2014; Flechas et al., 2012; Harris et al., 2006; Madison et al., 2017; Shaw et al., 2014), despite no evidence of its proficiency. We suggest that an understanding of the ecological interactions influencing the plated communities and the nature of the culture media will likely improve our ability to culture rare or previously uncultured microbes. Lastly, we would like to emphasize that, at least in our study species (and see Walke at al. 2015a), swabbing more individuals in a population is the best way to maximize culture collections, regardless of media type. These results can inform current efforts for developing a probiotic-based approach for amphibian conservation, and help to ensure that culture collections are capturing the majority of the important diversity in host-associated microbial systems.

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Table 1. List of indicator OTUs associated with the different culture media (LB, TSA, R2A and dR2A) or group of culture media types.

Culture media and groups of media	Indicator OTU ID	Phyla	Family	Genus	Indicator value index	*P* value
dR2A	156722	Proteobacteria	Burkholderiaceae	*Burkholderia*	0.78	0.001
	817982	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.50	0.03
	540793	Proteobacteria	Enterobacteriaceae	*Serratia*	0.45	0.047
	4315079	Firmicutes	Paenibacillaceae	*Paenibacillus*	0.43	0.017
	348478	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.42	0.022
	533999	Proteobacteria	Xanthomonadaceae	−	0.37	0.039
dR2A + R2A + TSA	654003	Proteobacteria	Phyllobacteriaceae	*Phyllobacterium*	0.90	0.01
LB + R2A + TSA	141365	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	0.86	0.035
R2A + dR2A	4449609	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.95	0.01
	241289	Proteobacteria	Xanthomonadaceae	*Luteibacter*	0.91	0.001
	5364	Proteobacteria	Rhizobiaceae	−	0.91	0.002
	denovo77494	Proteobacteria	Enterobacteriaceae	*Erwinia*	0.86	0.001
	denovo73459	Proteobacteria	Burkholderiaceae	*Burkholderia*	0.82	0.001
	denovo188538	Proteobacteria	Enterobacteriaceae	−	0.81	0.003
	denovo143862	Proteobacteria	Xanthomonadaceae	*Luteibacter*	0.77	0.001
	4421805	Actinobacteria	Microbacteriaceae	*Salinibacterium*	0.76	0.002
	denovo55330	Proteobacteria	Enterobacteriaceae	−	0.73	0.004
	denovo78257	Proteobacteria	Xanthomonadaceae	*Luteibacter*	0.73	0.001
	denovo22220	Proteobacteria	Enterobacteriaceae	−	0.73	0.001
	denovo196304	Proteobacteria	Burkholderiaceae	*Burkholderia*	0.72	0.002
	denovo54352	Proteobacteria	Enterobacteriaceae	−	0.72	0.001
	135993	Proteobacteria	Burkholderiaceae	*Burkholderia*	0.72	0.013
	denovo202891	Proteobacteria	Enterobacteriaceae	−	0.72	0.001
	denovo92894	Proteobacteria	Enterobacteriaceae	−	0.68	0.001
	4304056	Proteobacteria	Rhizobiaceae	−	0.68	0.003
	102915	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.68	0.012
	103410	Proteobacteria	Rhizobiaceae	*Rhizobium*	0.68	0.031
	denovo166093	Proteobacteria	Burkholderiaceae	−	0.63	0.002
	4311005	Proteobacteria	Burkholderiaceae	*Burkholderia*	0.59	0.007
	denovo67862	Proteobacteria	Xanthomonadaceae	*Luteibacter*	0.59	0.002
	denovo116903	Proteobacteria	Rhodobacteraceae	−	0.58	0.001
	4423410	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.58	0.015
	denovo167134	Proteobacteria	Enterobacteriaceae	−	0.57	0.027
	3180137	Proteobacteria	Rhodobacteraceae	−	0.56	0.004
	denovo145069	Actinobacteria	Nocardiaceae	*Rhodococcus*	0.55	0.019
	denovo191161	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	0.55	0.001
	denovo1424	Proteobacteria	Rhizobiaceae	*Rhizobium*	0.54	0.029
	218154	Proteobacteria	Rhizobiaceae	*Agrobacterium*	0.54	0.006
	denovo150449	Proteobacteria	Phyllobacteriaceae	*Phyllobacterium*	0.54	0.003
	1104627	Proteobacteria	Rhizobiaceae	*Rhizobium*	0.54	0.008
	673343	Proteobacteria	Alcaligenaceae	−	0.52	0.027
	662915	Proteobacteria	Aurantimonadaceae	−	0.50	0.009
	denovo83855	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.50	0.005
	543890	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.50	0.01

	125947	Proteobacteria	Burkholderiaceae	*Burkholderia*	0.50	0.022
	5162	Proteobacteria	Rhizobiaceae	−	0.50	0.036
	4479484	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.45	0.006
	4337890	Proteobacteria	Xanthomonadaceae	*Lysobacter*	0.45	0.045
	denovo164114	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.41	0.03
TSA	denovo1626	Proteobacteria	Rhizobiaceae	−	0.30	0.032

Indicator OTUs are listed in a decreasing order based on their indicator value index for a culture media or group of media. The indicator value index represents a measure of the association between an OTU and a culture media or group of media, and range from 0 to 1, where values close to 1 imply a relative stronger association.

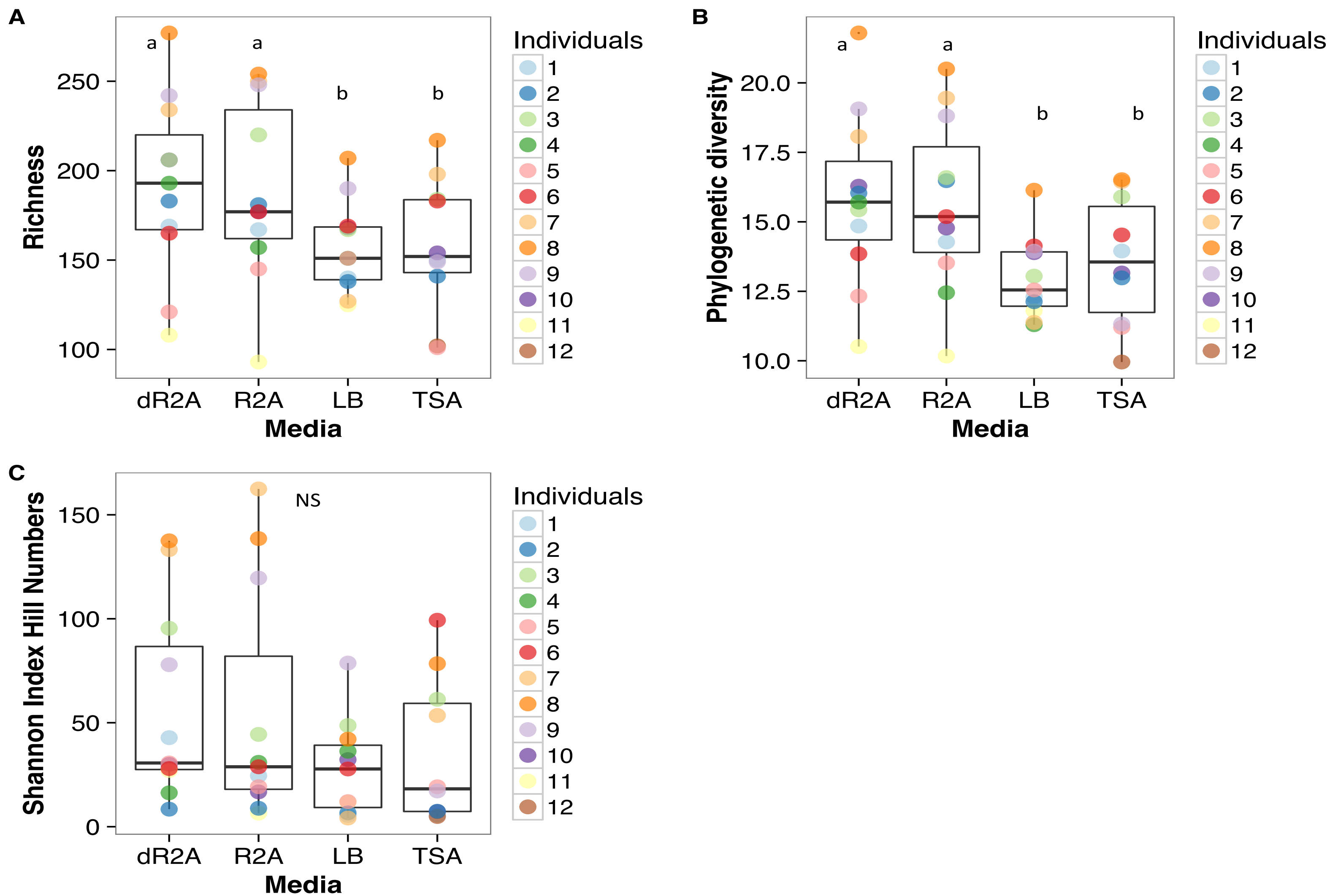

Figure 1. Alpha diversity estimates for the different metrics (**(A)**: OTU richness; **(B)**: Faith's phylogenetic diversity; **(C)**: Shannon Index) by culture media type. Alpha diversity estimates are color-coded at the toad (individual #). Boxplots represent the median, upper and lower quartile, and maximum and minimum values.

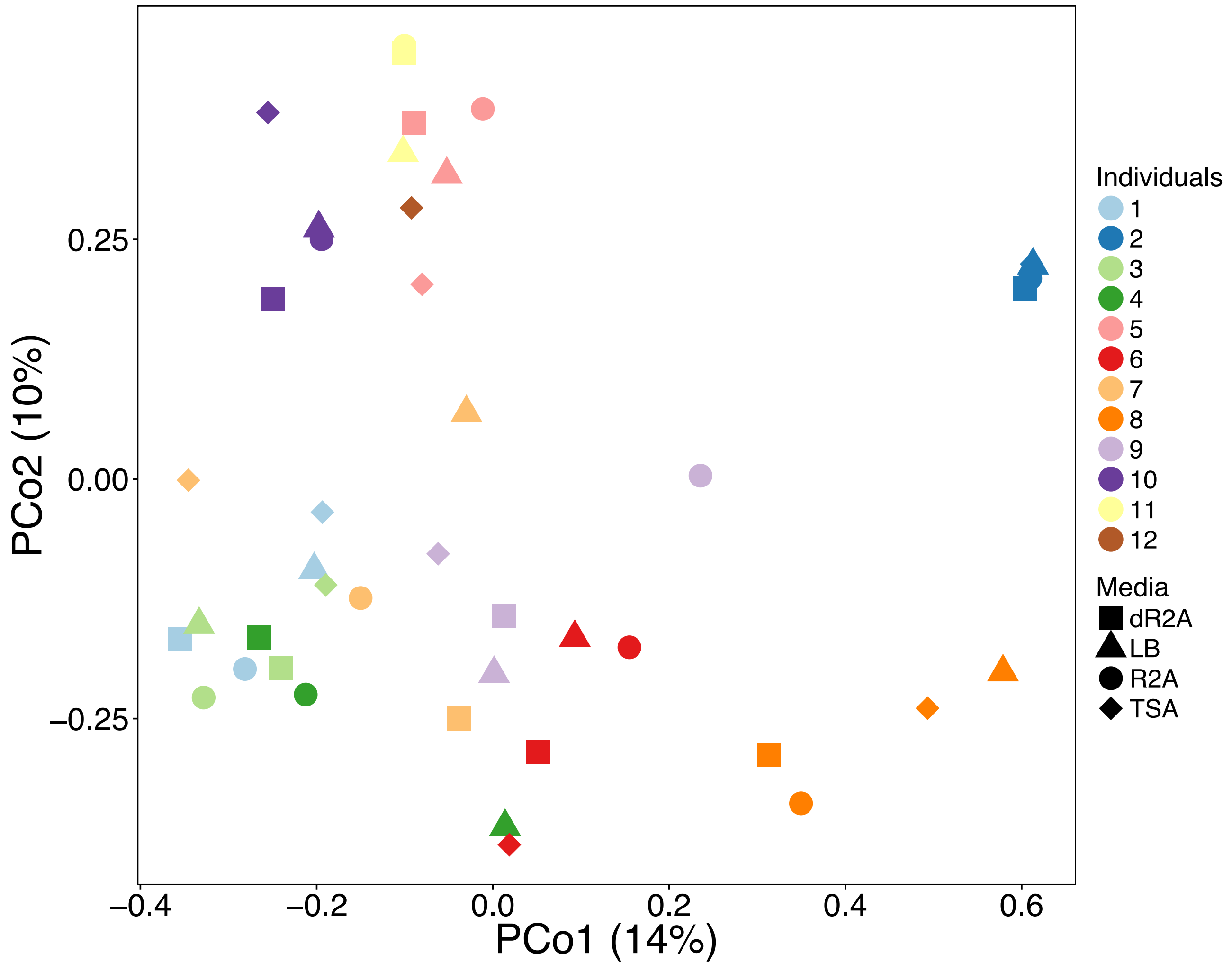

Figure 2. Beta diversity comparisons of the cultured bacterial communities using a principal coordinate analysis (PCoA) ordination based on Bray-Curtis dissimilarity distances. Points represent each plated community. Colors and shapes represent individual toads and culture media types, respectively.

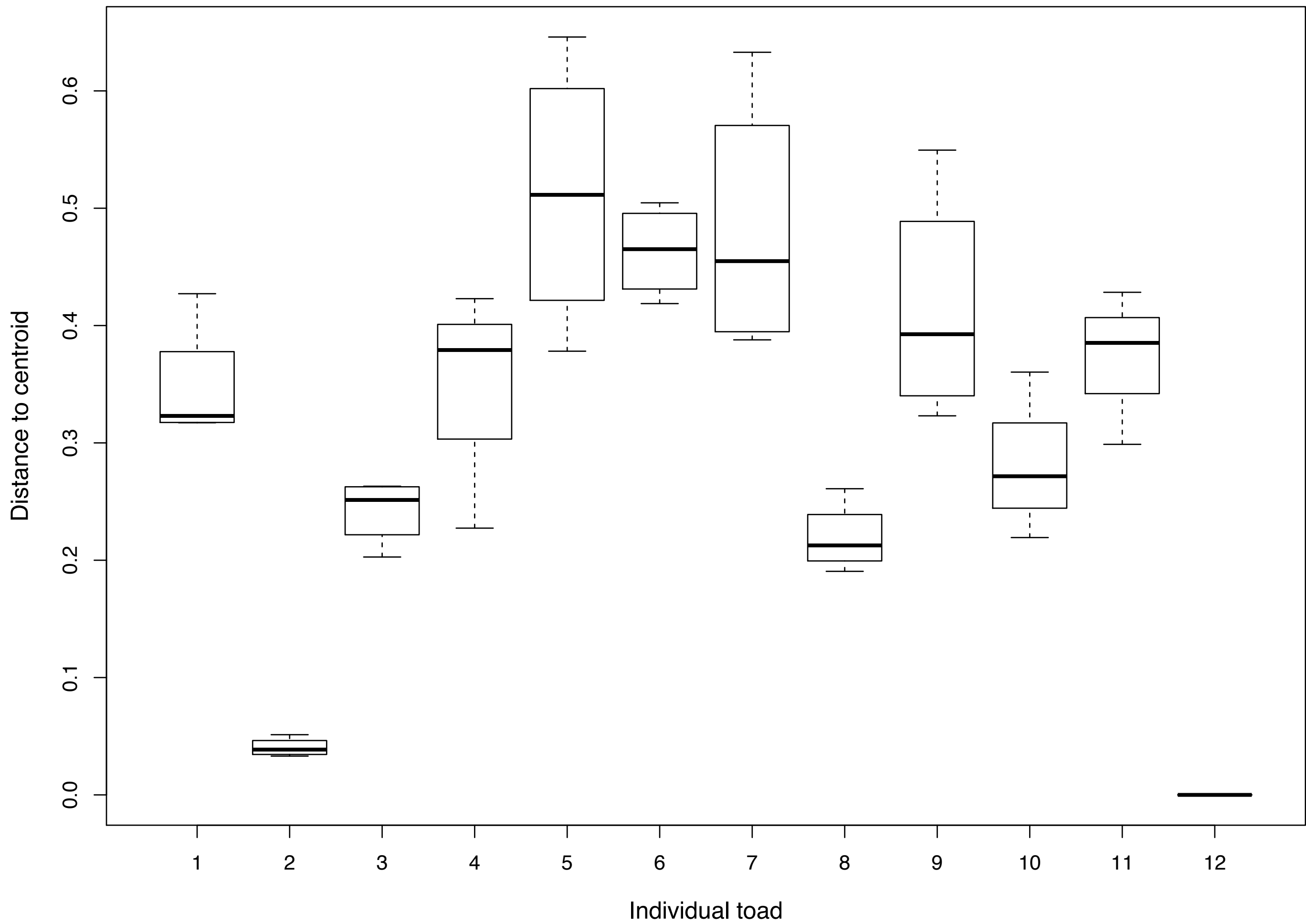

Figure 3. Dispersion among the plated bacterial communities from each individual toad. The analysis was conducted based on Bray-Curtis dissimilarity distances. The figure shows significant differences in dispersion among the individual toads. Boxplots represent the median, upper and lower quartile, and maximum and minimum values.

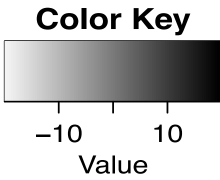

Figure 4. Heatmap of log-transformed relative abundances of the indicator OTUs associated with the different media types (LB, TSA, R2A and dR2A). Rows indicate each indicator OTU family and ID, and columns indicate the individual toads/replicates within each culture media type.

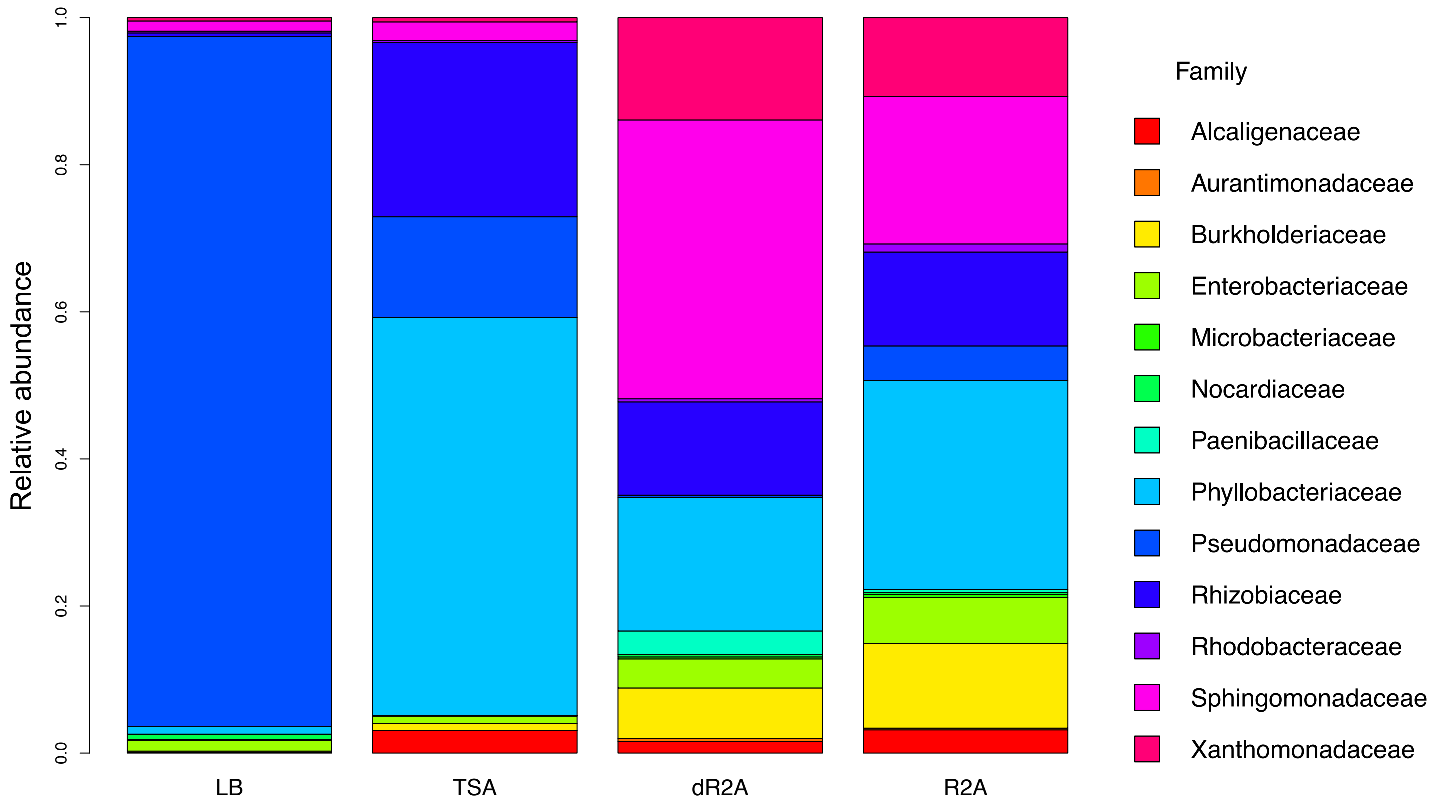

Figure 5. Stacked barplot showing the relative abundance, across toads/replicates within each culture media, of the taxonomic families of the indicator OTUs associated with the distinct culture media types.

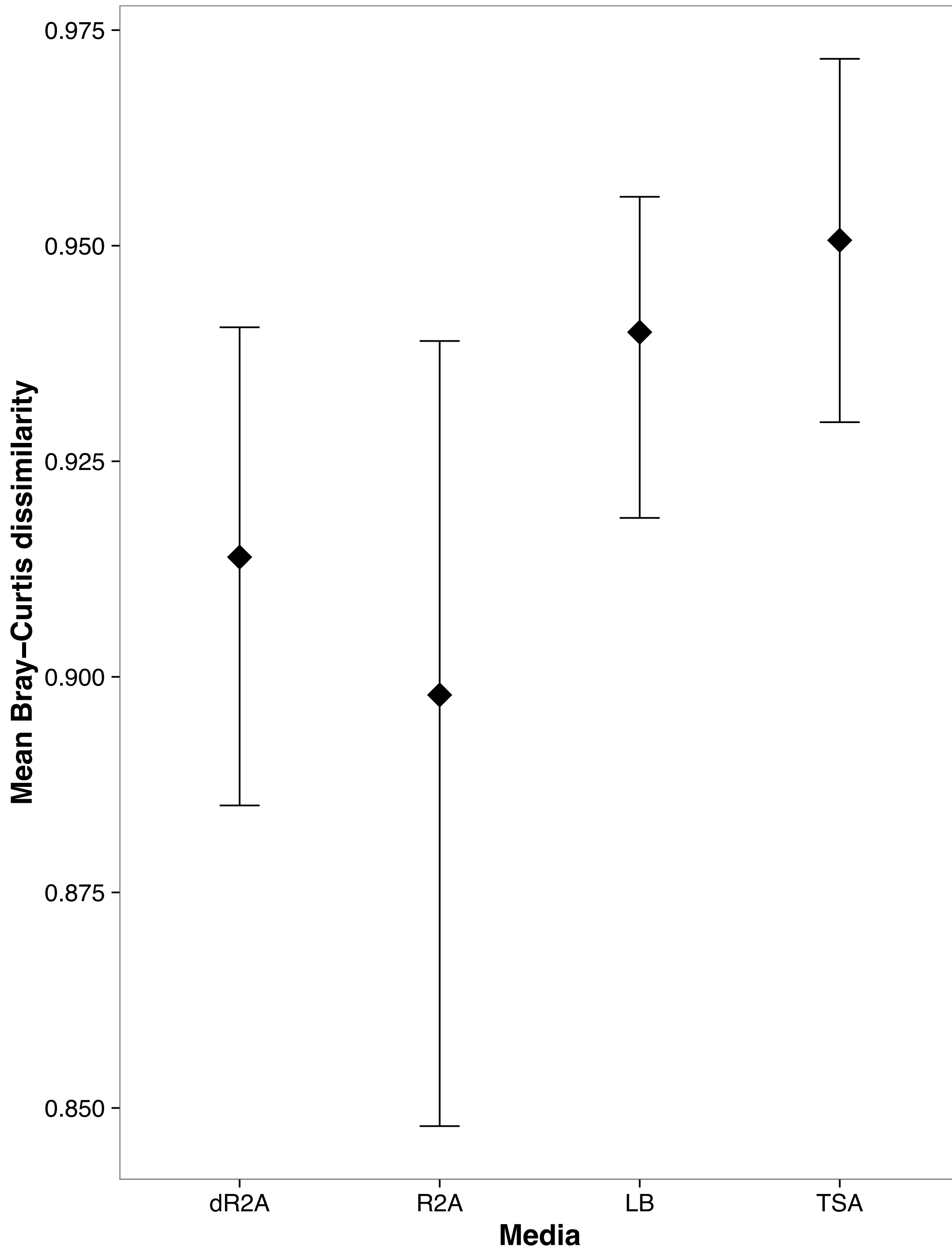

Figure 6. Mean and 95% CI of Bray-Curtis dissimilarity estimates between the cultured communities from the culture media types and the culture-independent bacterial communities from the skin swabs.

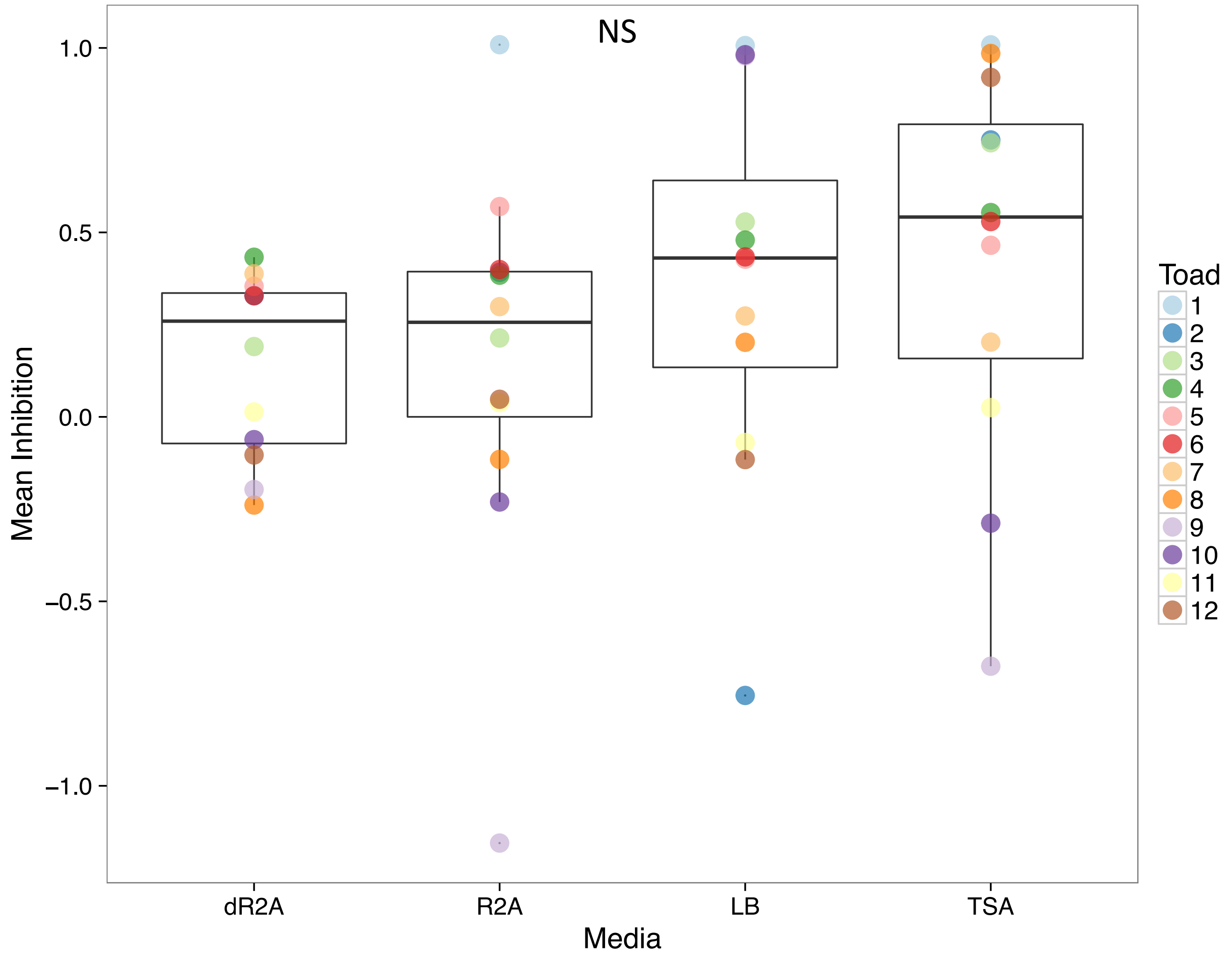

Figure 7. Mean inhibition estimates for each plated community by culture media. Estimates are color-coded at the toad level (individual #). Boxplots represent the median, upper and lower quartile, and maximum and minimum values. Inhibition estimates with negatives values suggest facilitation of Bd growth, while positive values suggest inhibition, with estimates = 1 representing a complete inhibition of Bd growth.

Chapter 4: Amphibian skin fungal communities vary across host species and regions, but do not correlate with infection by a pathogenic fungus

Abstract

Amphibian population declines caused by the pathogenic fungus, *Batrachochytrium dendrobatidis* (Bd), have prompted studies on the bacterial community that resides on amphibian skin. However, studies addressing the fungal portion of these symbiont communities have lagged behind. Using ITS1 amplicon sequencing, we examined the fungal portion of the skin microbiome of temperate and tropical amphibian species currently coexisting with Bd in nature. We assessed co-occurrence patterns between bacterial and fungal OTUs using a subset of samples for which bacterial 16S rRNA gene amplicon data were also available. We determined that the fungal communities were dominated by members of the phyla Ascomycota and Basidiomycota, and also by Chytridiomycota in the most aquatic amphibian species. Alpha diversity of the fungal communities differed across host species, and fungal community structure differed across species and regions. However, we did not find a correlation between fungal diversity or community structure and Bd infection. We identified a high proportion of positive bacterial-fungal co-occurrences, suggesting that positive interactions between these organisms occur in the amphibian skin microbiome. Understanding the ecology of amphibian skin fungal communities, and their interactions with bacteria, would complement our knowledge of the factors influencing community assembly and the overall function of these symbiont communities.

Introduction

The ubiquity, high diversity and function of host-associated microbes is changing our understanding about animal biology (McFall-Ngai *et al.*, 2013; Hird, 2017). This is due, in part, to the pivotal role that host-associated microbial communities can play in different aspects of host life history, overall host health, and fitness. For instance, host-associated microbial communities can aid the host in energy uptake and storage (Turnbaugh *et al.*, 2006), development (Diaz Heijtz *et al.*, 2011), maturation of the immune system (Chung *et al.*, 2012), behavior (Ezenwa *et al.*, 2012) and protection against pathogens (Flórez *et al.*, 2015). Much of this research has focused specifically on the bacteria that inhabit hosts, as they often form the bulk of microbial biomass (Huffnagle and Noverr, 2013).

In the last decade, amphibian skin bacterial communities have been subject to a large amount of research due to their potential protective role against pathogens. This body of research was primarily motivated by outbreaks of the disease chytridiomycosis, associated with population declines and extinctions of several amphibian species across the globe (Berger *et al.*, 1998; Bosch *et al.*, 2001; Lips *et al.*, 2006; Vredenburg *et al.*, 2010). Chytridiomycosis is caused by infections of the fungal skin pathogen *Batrachochytrium dendrobatidis* (Bd). Amphibian skin bacteria have been considered a promising area of research to develop a conservation strategy focused on using beneficial bacteria to mitigate Bd infections in endangered amphibians (Bletz *et al.*, 2013). This body of research has yielded important insights into the ecology of animal-associated microbes, and into their potential use in controlling emergent fungal diseases due to their capacity to interact with pathogenic agents, such as Bd (Walke and Belden, 2016). For instance, there is evidence that infections by Bd can alter the structure of amphibian skin bacterial communities (Jani and Briggs, 2014), and that the structure of the skin bacterial communities can be associated with susceptibility of the amphibian host to Bd infection (Lam *et*

al., 2010; Becker *et al.*, 2015; Rebollar *et al.*, 2016). However, studies characterizing and assessing the function of the fungal communities associated with amphibian skin are still scarce and lagging behind those on bacteria.

In general, in contrast to bacteria, the fungal diversity associated with animals, and their functional roles, remain largely understudied (Huffnagle and Noverr, 2013; Flórez *et al.*, 2015; Peay *et al.*, 2016). This is an important knowledge gap given that free-living fungi are highly diverse and are known to play important roles in ecological processes (Peay *et al.*, 2016). In addition, some have tight mutualistic and antagonistic interactions with plants (Tedersoo *et al.*, 2014). Recent research suggests that while the fungal component of animal-associated microbial communities is part of the rare biosphere [<0.1% of microbial cells] (Huffnagle and Noverr, 2013), its diversity, which includes novel lineages, is higher than expected (reviewed in Peay *et al.*, 2016). In addition, fungi are eukaryotes and are much larger in cell size than bacteria (>100 times larger), and thus, might contribute unique genetic and metabolic traits to their animal hosts (Underhill and Iliev, 2014), and influence the overall attributes of the microbial community. There is some evidence that fungi can engage in defensive symbiosis with animals. For example, fungus-farming insects (e.g. leaf-cutter ants, termites and bark beetles) can form symbiotic interactions with fungi, in which the symbiotic fungi provide protection to the insects and their fungal gardens against infections by fungal pathogens (reviewed in Flórez *et al.*, 2015). Moreover, as fungi can co-occur with bacteria within the host, research assessing the influence of fungi on the structure and function of bacterial communities is an emergent area of interest in host health, including in humans (Huffnagle and Noverr, 2013).

In an initial attempt to explore the non-bacterial microorganisms associated with amphibian skin, Kueneman *et al.* (2015) identified the microeukaryotes associated with the toad *Anaxyrus boreas.* They found that microeukaryote diversity was substantially higher in subadults and adults in contrast to tadpoles and juveniles. In addition, the authors determined that these communities of microeukaryotes in the subadults and adults were dominated by fungi. In another study, Kearns *et al.* (2017) found evidence of a potential defensive role against Bd by the fungal communities associated with the skin of captive amphibians. In addition, Kearns *et al.* (2017) determined that, in contrast to two bacterial probiotics (*Janthinobacterium lividum* and *Flavobacterium johnsoniae*), treating amphibians with a fungal probiotic (*Penicillium expansum*) induced a lower secretion rate of corticosterone (as a measure of stress), and did not suppress the production of antimicrobial peptides by the host. Thus, increasing our understanding of the ecology of amphibian skin fungal communities could provide important insights into the defensive role of symbiotic fungal communities and provide relevant information for current efforts in amphibian conservation.

Using data from field surveys, we assessed the diversity and natural variation of the skin fungal communities across eight species of free-living amphibians, representing two distinct regions (temperate and tropical). In addition, given the potential interaction of the fungal communities with host pathogens, we assessed whether infections by Bd influenced the diversity and community structure of the skin fungal communities. The study species currently co-exist with Bd in an endemic infection stage. Lastly, we examined the patterns of co-occurrence between the composition of bacterial and fungal communities associated with amphibian skin using a subset of samples for which we had both bacterial 16S rRNA and fungal ITS1 amplicon sequencing data.

Materials and Methods

Sample collection

We used previously extracted DNA from skin swabs (N = 85) from adults of eight amphibian species — four species from a temperate region and four from a tropical region (Table 1) — and characterized the fungal communities associated with their skin. In addition, to explore potential relationships between the composition of the fungal and bacterial communities, we used a subset of the samples (N = 63, Table 1) for which, with some exceptions, we had previously published bacterial 16S rRNA amplicon sequencing data (Belden *et al.*, 2015; Walke *et al.*, 2015).

The selection of samples aimed to capture a diversity of host species and to examine both Bd- and Bd+ individuals. The infection status used for the selection of samples (total Bd- individuals = 49, and Bd+ = 36) was based on prior results from Taqman real-time PCR [qPCR] (Rebollar *et al.*, 2014; Hughey *et al.*, 2014). However, there were slight discrepancies in the detection of Bd between prior qPCR results and the amplicon sequencing of the ITS1 region that we performed for this study. Thus, in our final analysis, the infection status and load were based on the amplicon data in order to stay consistent with the community data produced by amplicon sequencing, and in recognition of the possibility that the detection of Bd sequences might directly influence further statistical analysis. This resulted in small changes in the number of samples of Bd- and Bd+ within each study species (Table 1, total Bd- individuals = 46, and Bd+ = 39). The contrasting results between qPCR and amplicon sequencing might be explained, in part, by the relatively low infection loads observed in our samples based on the qPCR results: maximum infection load estimated was 388 zoospore genomic equivalents.

Individuals were initially sampled with skin swabs for bacterial community analysis and Bd detection between 2010-2013, during the spring and summer for the temperate species and during the rainy season for the tropical species. The skin swabs for all species were obtained using the same standardized swabbing technique (Hughey *et al.*, 2014; Rebollar *et al.*, 2014). Animal use was approved by the Institutional Animal Care and Use Committees of Virginia Tech and the Smithsonian Tropical Research Institute.

The amphibian species used in this study vary in their natural histories and reproductive modes. For instance, the species from the temperate region breed in ponds, which are permanent in the case of *Rana catesbeiana* and *Notophthalmus viridescens* or can also be ephemeral in the case of *Pseudacris crucifer* and *Anaxyrus americanus*, with the latter two species using more terrestrial habitats after metamorphosis. Of the species from the tropical region, *Agalychnis callidryas* and *Dendropsophus ebbraccatus* are arboreal species that breed in ponds, whereas *Silverstoneia flotator* and *Craugastor fitzingeri* are terrestrial species that can be found in the forest along streams and have terrestrial eggs, that in the case of *C. fitzingeri* hatch into froglets.

Sample and sequence data processing

DNA from the skin swabs was extracted following the manufacturer's protocol of the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) including an initial lysozyme incubation step at 37ºC for 1h, as described in previous studies (Hughey *et al.*, 2014; Walke *et al.*, 2015; Medina *et al.*, 2017). The extracted DNA was used as template for the detection and quantification of Bd mentioned earlier, and to conduct amplicon sequencing to determine the skin bacterial (previously done for a subset of the samples) and fungal (in the present study) communities.

The assessment of the skin fungal communities was conducted using amplicon sequencing of the ITS1 region with primers ITS1F and ITS2 (Bellemain *et al.*, 2010). Primers

included adapters and 2 bp linker sequences, and the reverse primer also included a unique 12 bp barcode sequence (Bellemain *et al.*, 2010). PCR reactions (25 µl) were prepared with 13 µl of PCR water (Mo Bio Laboratories, Carlsbad, CA, USA), 10 µl of 2.5x 5Prime HotMastermix, 0.5 µl of each primer (10 µM concentration), and 1 µl of template DNA. PCR reactions were conducted using a thermal cycling protocol with an initial temperature of 94ºC for 5 min, 35 cycles of 94ºC for 45 s, 50ºC for 45 s and 72ºC for 90 s, and a final extension at 72ºC for 10 min. Samples were run in triplicate and with a negative control, which lacked template DNA. PCR amplicons from triplicate reactions were pooled and visualized on 2% agarose gels. PCR amplicons were purified using the UltraClean PCR cleanup kit (Mo Bio Laboratories). Amplicon concentration was quantified using a Qubit 2.0 fluorometer (Invitrogen, USA) following the manufacturer's protocol prior to pooling in equimolar ratios into a composite sample. Fungal amplicons were sequenced on an Illumina MiSeq instrument using a 250 bp paired-end strategy at the Virginia Tech Biocomplexity Institute Sequencing Facility.

Sequencing reads associated with the skin fungal communities were processed as in Sun *et al.* (2017). Sequences were quality filtered and processed using the USEARCH pipeline (Edgar, 2010). Forward and reverse sequences were assembled based on a cut-off of at least 150 bp overlap. Assembled sequences were filtered based on a minimum length of 200 bp and an expected error per read lower than 0.5, which was calculated based on the quality score. Identification and removal of chimera sequences was conducted with UCHIME (Edgar *et al.*, 2011), and sequences that matched PhiX (~500,000), added to increase base diversity in Illumina sequencing, were removed from the dataset using Geneious (Biomatters, Ltd., version 8.1.8). Final sequences were assigned to operational taxonomic units (OTUs) based on a 97% similarity cut-off using the UCLUST method (Edgar, 2010), and aligned against the UNITE database (Koljalg *et al.*, 2013). Taxonomy was assigned using the RDP classifier (Wang *et al.*, 2007). The resulting OTU table had 2,390,653 sequences, with a sequencing depth per sample that ranged from 1,892 to 84,927 (mean sequences/sample: 28,125). The OTU table was rarefied to 1,850 reads/sample. This final dataset comprised 4,130 fungal OTUs across all samples, and the number of fungal OTUs per sample ranged from 18 to 303 (mean ± SD = 85 ± 74).

In this study, we also aimed to identify relationships between the compositions of the fungal and bacterial communities. For this analysis, we used a subset of the samples included in the fungal dataset (N = 63, Table 1). The bacterial data were compiled from 13 different prior Belden Lab Illumina Mi-Seq 16S rRNA gene amplicon sequencing runs using the QIIME pipeline. The *N. viridescens* samples and one *R. catesbeiana* sample included in the fungal community analysis were never processed in a 16S rRNA gene amplicon sequencing run, and thus they were excluded from this bacterial dataset. All bacterial samples were prepped for sequencing following our standard and published protocol (e.g. Belden *et al.*, 2015; Walke *et al.*, 2015). The samples were all sequenced at the Dana Farber Cancer Research Institute at Harvard University, Boston, MA, with 10% PhiX added to increase base diversity. Samples were run using a 250bp or 300bp single or paired-end strategy. Because of this variation, for the present study, we used only the forward reads of all samples. The bacterial raw Illumina 16S rRNA amplicon data were processed and quality filtered using QIIME (Caporaso *et al.*, 2010). We used the default settings for demultiplexing of the forward reads except that we allowed for no errors in the barcode and we set the required phred score at 20. This resulted in a loss of the *S. flotator* samples, as the forward read quality was too low, which resulted in almost all the reads being too short for inclusion in the dataset following this quality filtering. In Geneious (Biomatters, Ltd., version 8.1.8), we trimmed all sequences to 250bp and extracted those with 250 bp. The

remaining sequences were used to create the OTU table as described in Hughey *et al.* (2017). We rarefied all samples to a sequencing depth of 13,250, which resulted in the loss of one additional sample with low reads from the dataset. The final dataset consisted of 494 bacterial OTUs across 63 samples, with a range of 74-276 bacterial OTUs per sample (mean ± SD = 179 ± 45).

Data analysis

In addition to determining the taxonomic composition of the fungal communities associated with the skin of the host species, we assessed variation in alpha and beta diversity of the fungal communities across host species, region (temperate vs. tropical) and Bd infection status (+ or -). As there were differences in the communities among different hosts, we also identified the fungal OTUs driving those differences. Lastly, we assessed the relationship between the composition of the fungal and bacterial communities, and whether positive or negative interactions might predominate between these groups. Unless noted, all statistical analyses were conducted using R version 3.3.3 (R Core Team, 2017).

<u>Variation in fungal communities--Alpha and beta diversity analysis</u>

Alpha diversity comparisons across species, regions (temperate vs. tropical) and Bd infection status were conducted for richness and for the Shannon index transformed to Hill numbers (the effective number of species). Alpha diversity estimates were calculated for each sample using QIIME. To compare alpha diversity estimates of each metric across species, we fitted the estimates to generalized linear models (GLMs). In this analysis, for richness we used a negative binomial error distribution with the log link function to account for over dispersion, and the model was run using the function glm.nb from the package MASS (Venables and Ripley, 2002). For the Hill numbers calculated from the Shannon index estimates, we used a Gamma error distribution with the inverse link function and the model was run using the function glm from the R stats package. *P*-values for the models were estimated by comparing nested models using a likelihood ratio test (Zuur *et al.*, 2009; Belden *et al.*, 2015). Visual assessments of residual plots with model predictions verified the appropriateness of the error distributions. Pairwise comparisons were conducted by performing Tukey tests using the function glht from the package multcomp (Hothorn *et al.*, 2008), which includes multiple comparisons for generalized linear models.

Richness and Shannon index Hill numbers were also compared between regions (tropical vs. temperate). For this analysis, we fitted the diversity estimates to generalized linear mixed models (GLMMs), where the random factor in the model consisted of a random effect of "Site" nested within "Species" to account for the nestedness of samples from the same sites and species (Belden *et al.*, 2015). The error distributions mentioned above for richness and Shannon index Hill numbers were used to construct the models. The GLMMs were run using the functions glmer.nb and glmer for richness and Shannon index Hill numbers, respectively. Both of these functions are from the package lme4 (Bates *et al.*, 2014). *P*-values for the models were estimated as described above.

An assessment of a potential correlation between alpha diversity and Bd infection load (represented, at the sample level, by the total number of reads summed across the 23 fungal OTUs identified as Bd) was conducted using a generalized linear model (GLM) for richness and a linear model for Shannon index Hill numbers. For richness, the model contained the error distributions and link function described above, and used the function glm.nb from the MASS package (Venables and Ripley, 2002). In the case of Shannon index Hill numbers, the diversity

estimates were log-transformed to better meet the assumptions of normality and for better model fit, and the model was run using the lm function from the R stats package. The small number of samples containing a high number of Bd reads (>500 reads) relative to the number of samples with low numbers or no reads limited our ability to account for the random effect of the variables "Species" and/or "Site" in these models.

For beta diversity, we assessed the changes in the structure of the fungal communities across host species, regions (temperate vs. tropical) and Bd infection status. We calculated dissimilarity distances across samples based on Bray-Curtis (relative abundance) and Jaccard (presence/absence) dissimilarities. However, given consistent results using either distance metric, we only included the Bray-Curtis results. Statistical comparisons across variables were conducted with permutational multivariate analyses of variance (PERMANOVA; (Anderson, 2001). When comparing the changes in community structure between Bd infected and non-infected individuals, the argument "strata" was used to define the group within which to limit the permutations (i.e., amphibian species) in the PERMANOVA, due to the nestedness of the samples within species. In addition, to determine whether the variation in community structure between Bd infected and non-infected individuals was driven by Bd OTUs, we conducted a second analysis that excluded all Bd OTUs from the dataset. Dissimilarity distances were calculated using the function vegdist and the PERMANOVAs were conducted with the function adonis, both from the vegan package (Okansen *et al.*, 2016). Variation in community structure across variables was visualized using principal coordinates analyses (PCoA), with the function cmdscale, also from the vegan package.

Identification of abundant and host-specific fungal OTUs--Rank abundance curve and species indicator analysis

To identify the most abundant fungal OTUs based on their relative abundance, we calculated a rank abundance curve. An OTU rank abundance curve depicts the OTUs in order of absolute or relative abundance, specifically, from the highest rank to the lowest, where the most abundant OTU gets the highest rank and the least abundant gets the lowest rank. Given the high number of OTUs in the dataset with a low relative abundance, we performed a rank abundance curve using only the OTUs with a minimum relative abundance higher than 0.05 in at least one sample (199 OTUs). The rank abundance curve was calculated using the function radfit from the package vegan (Okansen *et al.*, 2016).

To identify fungal OTUs associated with specific amphibian species, we conducted a species indicator analysis using the function multipatt from the indicspecies package (De Cáceres and Legendre, 2009). This analysis calculates an indicator value that quantifies the association between OTUs and amphibian species, based on the relative abundance and relative frequency of each OTU, and uses a permutational approach to calculate the significance of the association based on an alpha value of 0.05 (De Cáceres and Legendre, 2009).

Exploring bacteria – fungi relationships

Using the subset of 63 samples for which we had bacterial 16S rRNA gene amplicon data, we assessed the potential relationship between the composition of the fungal and bacterial communities. We used a Mantel Test based on Jaccard dissimilarities to test for a correlation between the composition of the fungal and bacterial communities using the function mantel from the vegan package (Okansen *et al.*, 2016). Jaccard dissimilarities were used because we could

not combine bacterial and fungal relative abundance data in a single dataset, so we converted both datasets to presence/absence metrics for the combined analysis.

Potential interactions among bacterial and fungal OTUs were assessed with a co-occurrence network analysis. For this analysis, we combined the non-rarified bacterial and fungal datasets for the subset of 63 samples where both were available, which resulted in an OTU table with a total of 4391 OTUs. Given the decrease in sensitivity to detect correlations as zero counts increase (Weiss *et al.*, 2017), we removed the OTUs present in less than 10% (six) of the samples (removed 3685 OTUs) and the OTUs with less than a total sum of 10 reads across all the samples (removed 29 OTUs). The final OTU table had 677 OTUs. The correlations among OTUs were calculated using the default parameters of the method SparCC (Sparse Correlations for Compositional data, Friedman and Alm, 2012) in Python (version 2.7.10). This method uses a permutation-based approach (100 permutations in this case) to calculate bootstrapped pseudo *P*-values for each pairwise correlation. Only significant correlations (alpha value = 0.05) with values smaller than -0.35 and larger than 0.35 were used (Kueneman *et al.*, 2017). Results were visualized using the igraph package (Csardi and Nepusz, 2006).

Results

Seven phyla comprised the amphibian skin fungal communities (Fig. 1a). However, these communities were dominated by OTUs from the phylum Ascomycota, with a total proportional abundance ranging from 0.43 – 0.64 across the amphibian species (Fig. 1a). The phylum Basidiomycota was the second most abundant on most amphibian species, with the exception of *N. viridescens*, with a proportional abundance ranging from 0.12 – 0.38 (Fig. 1a). Interestingly, on *N. viridescens* the second most abundant phylum was Chytridiomycota, which included 151 OTUs other than Bd (Figure 1a) and had a proportional abundance of 0.22 in this species. We also determined that the occurrence and relative abundance of fungal families varied substantially across amphibian species, although one family, Davidiellaceae, was present on all the species when accounting for abundant OTUs only, and had a proportional abundance from 0.04 – 0.51 (Fig. 1b). In terms of OTU relative abundance, there was a right skewed distribution, where a few OTUs were highly abundant relative to the number of rare ones (Fig. 2). The two most abundant OTUs (OTU_1 and OTU_2, both from the phylum Ascomycota) were the only ones present in over half of the samples (OTU_1 present in 86% of the samples and OTU_2 in 74%). OTU_1, the most abundant and prevalent OTU, was identified as a fungal species from the genus *Cladosporium* based on sequence alignment of this OTU against the NCBI database using BLAST (Basic Local Alignment Search Tool; hits results: score = 459, query cover 100% and *e*-value = 2e-125). OTU_2, the second most abundant, was classified as an "uncultured fungus" from the phylum Ascomycota based on BLAST and the NCBI database (hits results: score = 459, query cover 99% and *e*-value = 2e-125). These two OTUs were not identified beyond the kingdom or class level using the database UNITE.

We found that richness and Shannon index Hill numbers differed across amphibian species (richness: Chisq = 212.35, $P < 0.001$; Shannon index Hill numbers: Chisq = 90.63, $P < 0.001$). Amphibian species with terrestrial habitats (*C. fitzingeri*, *S. flotator* and *A. americanus*) seemed to have a higher diversity (Fig. 3ab; see Table S1 for statistical pairwise comparisons). Despite differences across species in alpha diversity, we found no significant differences between the tropical and temperate species in overall richness and Shannon index Hill numbers (richness: Chisq = 1.56, $P = 0.21$; Shannon index Hill numbers: Chisq = 3.37, $P = 0.07$), which suggests that the amphibian host-species plays a more important role in determining the richness

and evenness of the skin fungal communities than biogeographic region (Fig. 3ab). Bd OTU read number (as a proxy for infection load) was not correlated with either richness or Shannon index Hill numbers (Fig. 3c; richness: back-transformed slope = 0.99, SE = 1.95E-4, $R^2 = 0.006$, $P = 0.42$; Shannon index Hill numbers: slope = 4.306E-4, SE = 3.382E-4, $R^2 = 0.007$, $P = 0.21$).

Skin fungal community structure differed across species, regions and Bd infection status (species: pseudo-F = 2.74, $R^2 = 0.20$, $P < 0.001$, Fig. 4; region: pseudo-F = 3.69, $R^2 = 0.04$, $P < 0.001$; Bd infection status: pseudo-F = 2.35, $R^2 = 0.03$, $P < 0.001$). However, the factor 'species' explained substantially more variation relative to the factors 'region' and 'Bd infection status', as represented by the R-squared values. The differences in the structure of the skin fungal communities across Bd infection status seemed to be primarily driven by the presence of Bd OTUs at high relative abundances and not to large changes in the composition or relative abundance of other fungal OTUs, as demonstrated by the results from the analysis excluding the Bd OTUs (Fig. 4b, pseudo-F = 1.13, $R^2 = 0.01$; $P = 0.052$). Lastly, there were 55 indicator OTUs (including eight Bd OTUs) that defined the amphibian host species and/or group of species. Of these, 32 were each associated with a single, but not the same, amphibian species (Table 2).

Using the 63 samples for which we also had bacterial 16S rRNA gene amplicon data, we found a potential correlation between the composition of the fungal and bacterial communities (Jaccard, Mantel r statistic = 0.23, $P = 0.001$). In addition, we identified 127 significant pairwise correlations between OTUs in the co-occurrence network analysis (Figure 5; number of nodes: 211; number of edges: 254; average degree: 1.20; connectedness: 0.0057), which included correlations between bacteria (65% of interactions), bacteria–fungi (33%) and fungi (2%). Interestingly, bacterial OTUs from the phylum Proteobacteria were involved in most of the significant correlations (68%, Figure 5). The proportion of positive and negative correlations was similar between correlated bacterial OTUs (positive correlations: 48%; negative: 52%); however, positive correlations were predominant between correlated bacterial – fungal OTUs (positive correlations: 79%; negative: 21%; Fig. 5). While there were few significant correlations between fungal OTUs, all of them were positive.

Discussion

The fungal portion of the skin microbiome of the amphibian species in this study was dominated by members of the phyla Ascomycota and Basidiomycota, and, in one instance, by non-Bd members of the phylum Chytridiomycota. These are also the major fungal phyla in most natural systems. For instance, the phyla Ascomycota, Basidiomycota and Chytridiomycota were identified, among others, as the taxa with the highest number of OTUs in soil samples from around the world (Tedersoo *et al.*, 2014). In the case of the phyla Ascomycota and Basidiomycota, they include a diverse group of species involved in mycorrhizal and endophytic symbiosis (Peay *et al.*, 2016). Moreover, animal-associated fungal communities, including human-associated communities, are also dominated by species from the phyla Ascomycota and Basidiomycota (Huffnagle and Noverr, 2013; Peay *et al.*, 2016). However, while the fungal diversity we identified on the skin of amphibians might be comprised of fungi involved in commensal and mutualistic interactions, there are also likely transient spores (Peay *et al.*, 2016).

We also determined that the newts, *N. viridescens,* harbored a higher occurrence of non-Bd members of the phylum Chytridiomycota relative to the other amphibian species. Considering that chytrids can be abundant in freshwater systems (James *et al.*, 2006), their higher occurrence on *N. viridescens* might be explained, in part, by an increase in exposure to these fungi in the aquatic habitat of the adult stage of this species. For instance, an OTU

identified as a chytrid from the genus *Angulomyces*, a taxon found in multiple aquatic habitats from distinct biogeographic regions (Letcher *et al.*, 2008; Davis *et al.*, 2013), was determined to be an indicator OTU associated with *N. viridescens*. *N. viridescens* also harbored other chytrids representing more terrestrial taxa, such as the genera *Spizellomyces* (also identified as an indicator OTU) and *Powellomyces*, which are usually found in soil and near the shore of aquatic habitats (Wakefield *et al.*, 2010). This result suggests the composition of the fungal portion of the amphibian skin microbiome can be influenced by the environmental species pool, which has also been observed for amphibian skin bacteria (Muletz *et al.*, 2012; Loudon *et al.*, 2013; Walke *et al.*, 2014). Within this context, we also observed higher fungal community richness in the host species that are more strongly associated with terrestrial habitats, such as the forest floor (*C. fitzingeri*, *S. flotator* and *A. americanus*). This observed diversity pattern might have resulted from the constant exposure of these amphibian species to substrates with very high fungal diversity, such as soil (Peay *et al.*, 2016). While we did not systematically test for the effect of host ecology on the amphibian skin fungal communities, our results are consistent with previous work by Bletz *et al.* (2017) on amphibian skin bacterial communities, where terrestrial host species harbored a higher richness of bacterial OTUs compared to the arboreal and aquatic species. Given the role of environmental microbes as a source pool for maintaining the bacterial diversity of the amphibian skin communities (Loudon *et al.*, 2013; Bletz *et al.*, 2017; Muletz-Wolz *et al.*, 2017), it is also possible that host species associations with different habitats leads to exposure to distinct microbial pools, producing some of the observed variation in the fungal diversity of the skin communities.

With regard to the dominant members of the amphibian skin fungal communities at the phylum level, our results are consistent with prior work. For instance, a study using captive Dendrobatid frogs suggested that all four species harbored a large and diverse group of members of the phylum Ascomycota (Kearns *et al.*, 2017). Moreover, within the context of infection by Bd, Kearns *et al.* (2017) determined that the amphibian species thought to be resistant to Bd infection (i.e. *Dendrobates leucomelas*) harbored more Bd-inhibitory fungal taxa from the phylum Ascomycota, as compared to the more susceptible host species. Further research testing Ascomycota isolates from wild amphibians against Bd provides a promising avenue to elucidate the potential for defensive symbiosis between animals and fungi, which remains largely understudied (reviewed in Flórez *et al.*, 2015). On the other hand, Kearns *et al.* (2017) also identified several taxa in the phylum Basidiomycota with the ability to facilitate the growth of Bd zoospores. Perhaps the balance of these two major fungal phyla is important in host response to Bd.

In contrast to the findings of Kearns *et al.* (2017), we did not find a strong relationship between Bd infection and fungal community diversity or structure in the free-living amphibians sampled in our study. However, in our network analysis, we found significant correlations, both negative and positive, between Bd OTUs and both bacterial and fungal OTUs, including bacterial OTUs from the phylum Proteobacteria (families: Enterobacteriaceae, Moraxellaceae and Sphingomonadeceae), Firmicutes (family: Enterococcaceae), Cyanobacteria, and OTUs from the fungal phylum Ascomycota (family: Nectriaceae). These significant individual correlations suggest there could still be important interactions between Bd and specific bacterial and fungal taxa that alter disease outcomes, as has been suggested previously (Kueneman *et al.*, 2017). However, we also had a small number of samples with high Bd reads, potentially because the species used in our study are thought to be tolerant or resistant to Bd infection and have extant populations co-existing with Bd at an enzootic stage (Crawford *et al.*, 2010; Rebollar *et al.*,

2014; Hughey *et al.*, 2014). Thus, it may be more difficult with our dataset of wild-sampled amphibians to detect small effects of Bd on community-level metrics.

Relative to diversity patterns of bacterial communities, free-living fungal communities seem to exhibit a higher degree of heterogeneity at local and global scales (Martiny *et al.*, 2011; Baldrian *et al.*, 2012; Koljalg *et al.*, 2013; Brown and Jumpponen, 2013; Peay *et al.*, 2016), which results in substantial changes in community composition across habitats (Peay *et al.*, 2016). Within this context, in our study we observed that the composition of the fungal communities also tended to vary substantially at the family level across amphibian host-species. One exception to this was the family Davidiellaceae, which was present in all amphibian species. This family was represented by only one OTU (OTU_1), and it was the most frequent and abundant OTU in our dataset. Interestingly, this OTU was identified as a fungus from the genus *Cladosporium*. Members of this genus have been associated with mycoses in amphibians, such as mycotic dermatitis and mycotic myositis (Taylor, 2001). For instance, the species *Cladosporium cladosporoides* has been associated with the disease chromomycosis, a type of mycotic dermatitis, on invasive *R. catesbeiana* in Korea (Kim *et al.*, 2008). In addition, captive amphibians in zoos can also harbor fungi from the genus *Cladosporium* at high relative abundances (Kearns *et al.*, 2017), and in some cases these infections result in cases of chromomycosis (Taylor, 2001). Importantly, although we detected a fungal OTU associated with the genus *Cladosporium* across a broad range of amphibians, including *R. catesbeiana,* in which this OTU had the highest relative abundance, we have no evidence that the amphibians sampled in our study had clinical symptoms of mycotic dermatitis. However, our study does provide evidence of how conspicuous and widespread this genus can be across amphibian hosts.

Host-associated microbial communities are subject to strong selective pressure posed by the host via different deterministic processes, which can lead to host species-specific symbiotic communities (Adair and Douglas, 2017). For instance, similar to host-associated bacterial communities, host-specific fungal communities have been observed across a wide range of hosts, such as plants, marine invertebrates and terrestrial vertebrates (Huffnagle and Noverr, 2013; Yarden, 2014; Christian *et al.*, 2015; Peay *et al.*, 2016). Our results provide further support for host-specific fungal communities in amphibians (Kearns *et al.*, 2017). Host-associated traits, that either preclude or enhance the colonization of specific microbes (Gallo and Hooper, 2012; Franzenburg *et al.*, 2013), are likely to play a pivotal role in shaping these symbiotic fungal communities. In the amphibian skin microbiome system, host specificity of the bacterial communities has also been observed across a broad range of amphibian species (Kueneman *et al.*, 2013; Walke *et al.*, 2014; Belden *et al.*, 2015; Rebollar *et al.*, 2016; Prado-Irwin *et al.*, 2017; Hernández-Gómez *et al.*, 2017). Interestingly, from a broader scale, relatively little variation in the fungal communities we studied was explained by the biogeographic region of the amphibian species. This suggests that amphibian skin, which contains a combination of mucopolysaccharides, proteins and peptides, might provide a unique habitat for some fungal taxonomic groups in particular (i.e. habitat filtering). This observation is further supported by the pattern observed in amphibian bacterial communities, where the same phyla dominate the bacterial portion of the skin microbiome of amphibians from distinct regions of the world (Kueneman *et al.*, 2013; Belden *et al.*, 2015; Sabino-Pinto *et al.*, 2016; Bletz *et al.*, 2017).

Microbial interactions can also influence the subset of microbes that colonize and constitute a host-associated community. In fact, these interactions are generally considered as a deterministic process shaping the diversity and structure of symbiotic communities (Adair and Douglas, 2017), specifically via positive, negative or neutral feedbacks on the microbial species

involved in them (Faust and Raes, 2012; Adair and Douglas, 2017). Consistent with this, using the subset of samples for which we had both 16S rRNA and ITS1 amplicon sequencing data, we determined a potential correlation between the composition of the bacterial and fungal communities. In addition, based on the co-occurrence network analysis that included the same subset of samples, we identified multiple statistically significant correlations among OTUs, which included correlations between bacteria and fungi. Thus, cross-Domain interactions might be occurring on the skin of amphibians, which is consistent with a previous study that assessed the dynamics of the skin microbiome of an amphibian species across life stages (Kueneman *et al.*, 2015). Moreover, our results further support the determination by Kueneman *et al.* (2015) that OTUs from the phylum Proteobacteria (class: Betaproteobacteria) were correlated with a large fraction of the network, which included both bacterial and fungal OTUs. Specifically, we found that Proteobacteria OTUs, besides being correlated with multiple other bacterial taxa, were also correlated with most of the fungal OTUs identified in the co-occurrence network. This is relevant considering that bacteria – fungi interactions can influence different aspects of a microbe's fitness on the host, such as colonization, survival and pathogenesis, which can subsequently have an effect on the overall function of the symbiotic community (Wargo and Hogan, 2006). However, while symbiotic interactions between fungi and Proteobacteria have been identified in a variety of systems (reviewed in Wargo and Hogan, 2006), this co-occurrence pattern might be explained, in part, by the fact that Proteobacteria was one of the most abundant phyla in the skin microbiome of the amphibian species used in this study (Belden *et al.*, 2015; Walke *et al.*, 2015; Medina *et al.*, 2017). In addition, differences in library preparation and sequence processing between the bacterial and fungal datasets could have influenced the results and interpretation of the co-occurrence analysis.

While it is known that bacteria and fungi can be involved in antagonistic interactions, there is growing evidence that these organisms engage in many positive interactions (Artursson *et al.*, 2006; Wargo and Hogan, 2006; Frey-Klett *et al.*, 2007). For instance, bacterial products can stimulate fungal growth (e.g. auxofuran; Riedlinger *et al.*, 2006), can activate genes involved in the production of fungal secondary metabolites (Schroeckh *et al.*, 2009), and promote fungal survival (e.g. through protection against antibiotics; Wargo and Hogan, 2006). With this in mind, the high percentage (79%) of positive correlations between bacterial and fungal OTUs found in our study might suggest that positive interactions between these organisms could occur in the amphibian skin microbiome. Experimental work manipulating the composition of mixed cultures will be required to validate the biological meaning of patterns of co-occurrence we identified in the network analysis. Within the context of Bd infection, bacteria – fungi interactions might add another layer of complexity to studies focusing on the mechanisms shaping skin community assembly and overall function against Bd, especially considering that Kearns *et al.* (2017) found that a higher proportion of the amphibian skin fungal community inhibited the growth of Bd compared to that of the bacterial community. In addition, future research using a metagenomic or transcriptomic approach could identify genes and metabolic pathways potentially involved in host-fungi interactions (Sharpton, 2014), which would help to elucidate to what degree the amphibian skin fungal communities are symbiotic.

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Table 1. Amphibian species used in the study, their region of occurrence, locality, sampling dates and sample sizes for the fungal and bacterial datasets.

Region	Species	Province (Panamá)/State-County (USA)	Sampling period	Samples infection status (based on ITS1 Illumina reads)		Fungal dataset sample size	Bacterial dataset sample size
				Positive	Negative		
Tropical (Panamá)	*Agalychnis callidryas* (family: Hylidae)	Panamá	July – Sept 2012	7	1	8	8[a]
		Colón	Jul 2013	2	6	8	8
	Dendropsophus ebraccatus (family: Hylidae)	Panamá	July – Sept 2012	2	5	7	7[a]
		Colón	Jul 2013	2	6	8	8
	Silverstoneia flotator (family: Dendrobatidae)	Coclé	Jun 2013	4	2	6	–
		Coclé	Jun 2013	0	6	6	–
	Craugastor fitzingeri (family: Craugastoridae)	Panamá	July – Sept 2012	3	4	7	7[a]
Temperate (United States)	*Rana catesbeiana* (family: Ranidae)	Virginia - Giles	Aug 2010	3	5	8	7[b]
	Pseudacris crucifer (family: Hylidae)	Virginia - Bedford	Mar 2012	7	1	8	7
		Virginia - Giles	Mar 2012	4	2	6	6
	Notophthalmus viridescens (family: Salamandridae)	Virginia - Giles	Apr 2012	4	4	8	–
	Anaxyrus americanus (family: Bufonidae)	Virginia - Craig	Mar 2012	1	4	5	5
						85	**63**

Bacterial 16S rRNA amplicon sequencing data published in: [a]Belden *et al.* (2015) and [b]Walke *et al.* (2015).

Table 2. List of indicator OTUs (including eight Bd OTUs) that defined the amphibian host species and/or group of species.

Amphibian-host species/group of species	Indicator OTU ID	Phyla	Order	Genus	Indicator index	*P* value
Agalychnis callidryas	OTU_52	Basidiomycota	Filobasidiales	–	0.30	0.012
	OTU_69	Basidiomycota	Cystofilobasidiales	*Guehomyces*	0.22	0.039
Craugastor fitzingeri	OTU_26	Ascomycota	Hypocreales	*Calonectria*	0.59	0.001
	OTU_254	Ascomycota	Hypocreales	*Calonectria*	0.46	0.001
	OTU_91	Ascomycota	Xylariales	–	0.43	0.002
	OTU_17	Ascomycota	Xylariales	–	0.42	0.004
	OTU_87	Ascomycota	Saccharomycetales	*Yamadazyma*	0.34	0.015
	OTU_128	Zygomycota	Mortierellales	*Mortierella*	0.29	0.011
	OTU_35	Basidiomycota	Agaricales	–	0.29	0.006
Silverstoneia flotator	OTU_45	Ascomycota	Sordariomycetes_ord_Incertae_sedis	*Phialemoniopsis*	0.32	0.022
	OTU_192	Ascomycota	Hypocreales	*Nectria*	0.25	0.028
Anaxyrus americanus	OTU_44	Unclassified fungi	–	–	0.80	0.001
	OTU_78	Basidiomycota	Cystofilobasidiales	*Mrakiella*	0.79	0.001
	OTU_120	Ascomycota	Capnodiales	*Mycosphaerella*	0.74	0.001
	OTU_500	Ascomycota	Chaetothyriales	*Phaeococcomyces*	0.60	0.001
	OTU_11	Basidiomycota	Polyporales	*Trametes*	0.59	0.002
	OTU_123	Ascomycota	Helotiales	*Oculimacula*	0.40	0.004
	OTU_154	Basidiomycota	Cantharellales	–	0.36	0.007
Notophthalmus viridescens	OTU_19	Chytridiomycota	Rhizophydiales	*Angulomyces*	1.00	0.001
	OTU_34	Chytridiomycota	Spizellomycetales	*Spizellomyces*	0.88	0.001
	OTU_93	Basidiomycota	Sebacinales	–	0.75	0.001
	OTU_86	Ascomycota	Helotiales	–	0.46	0.003
	OTU_53	Chytridiomycota	Rhizophydiales	*Batrachochytrium*	0.41	0.009
Pseudacris crucifer	OTU_169	Unclassified fungi	–	–	0.29	0.01
Rana catesbeiana	OTU_16	Ascomycota	Capnodiales	*Toxicocladosporium*	0.75	0.001
	OTU_60	Ascomycota	Pleosporales	–	0.73	0.001
	OTU_6	Ascomycota	Capnodiales	*Toxicocladosporium*	0.63	0.001
	OTU_43	Chytridiomycota	Blastocladiales	*Catenaria*	0.50	0.001
	OTU_117	Glomeromycota	Diversisporales	*Acaulospora*	0.47	0.007
	OTU_223	Ascomycota	Capnodiales	*Cladosporium*	0.45	0.001

	OTU_59	Ascomycota	Pleosporales	*Leptosphaeria*	0.36	0.011
	OTU_252	Basidiomycota	Sebacinales	*Serendipita*	0.25	0.026
C. fitzingeri and D. ebraccatus	OTU_30	Ascomycota	Saccharomycetales	*Candida*	0.52	0.005
C. fitzingeri and S. flotator	OTU_71	Ascomycota	Dothideales	–	0.42	0.01
	OTU_112	Unclassified fungi	–	–	0.26	0.016
S. flotator and A. americanus	OTU_57	Ascomycota	Hypocreales	*Simplicillium*	0.29	0.031
A. americanus and R. catesbeiana	OTU_21	Ascomycota	–	–	0.61	0.001
N.viridescens and P. crucifer	OTU_4	Chytridiomycota	Rhizophydiales	*Batrachochytrium*	0.38	0.034
A. callidryas, C. fitzingeri and S. flotator	OTU_29	Ascomycota	Xylariales	*Monographella*	0.45	0.031
C. fitzingeri, D. ebraccatus and S. flotator	OTU_18	Basidiomycota	Sporidiobolales	*Rhodotorula*	0.48	0.006
	OTU_107	Ascomycota	Hyprocreales	*Verticillium*	0.35	0.022
C. fitzingeri, N. viridescens and P. crucifer	OTU_2016	Chytridiomycota	Rhizophydiales	*Batrachochytrium*	0.34	0.012
	OTU_2021	Chytridiomycota	Rhizophydiales	*Batrachochytrium*	0.29	0.04
C. fitzingeri, S. flotator, N. viridescens and P. crucifer	OTU_7	Chytridiomycota	Rhizophydiales	*Batrachochytrium*	0.48	0.035
	OTU_1337	Chytridiomycota	Rhizophydiales	*Batrachochytrium*	0.39	0.01
	OTU_9	Chytridiomycota	Rhizophydiales	*Batrachochytrium*	0.39	0.027
	OTU_92	Chytridiomycota	Rhizophydiales	*Batrachochytrium*	0.34	0.03
A. americanus, N. viridescens, P. crucifer and R. catesbeiana	OTU_8	Ascomycota	Pleosporales	–	0.48	0.002
A. callidryas, C. fitzingeri, D. ebraccatus, S. flotator and R. catesbeiana	OTU_209	Ascomycota	Pleosporales	–	0.43	0.02
A. callidryas, D. ebraccatus, A. americanus,	OTU_41	Basidiomycota	Tremellales	*Cryptococcus*	0.46	0.023

N. virisdescens and P. crucifer						
A. callidryas, C. fitzingeri, D. ebraccatus, S. flotator, A. americanus and N. virisdescens	OTU_2	Ascomycota	Hyprocreales	−	1.00	0.001

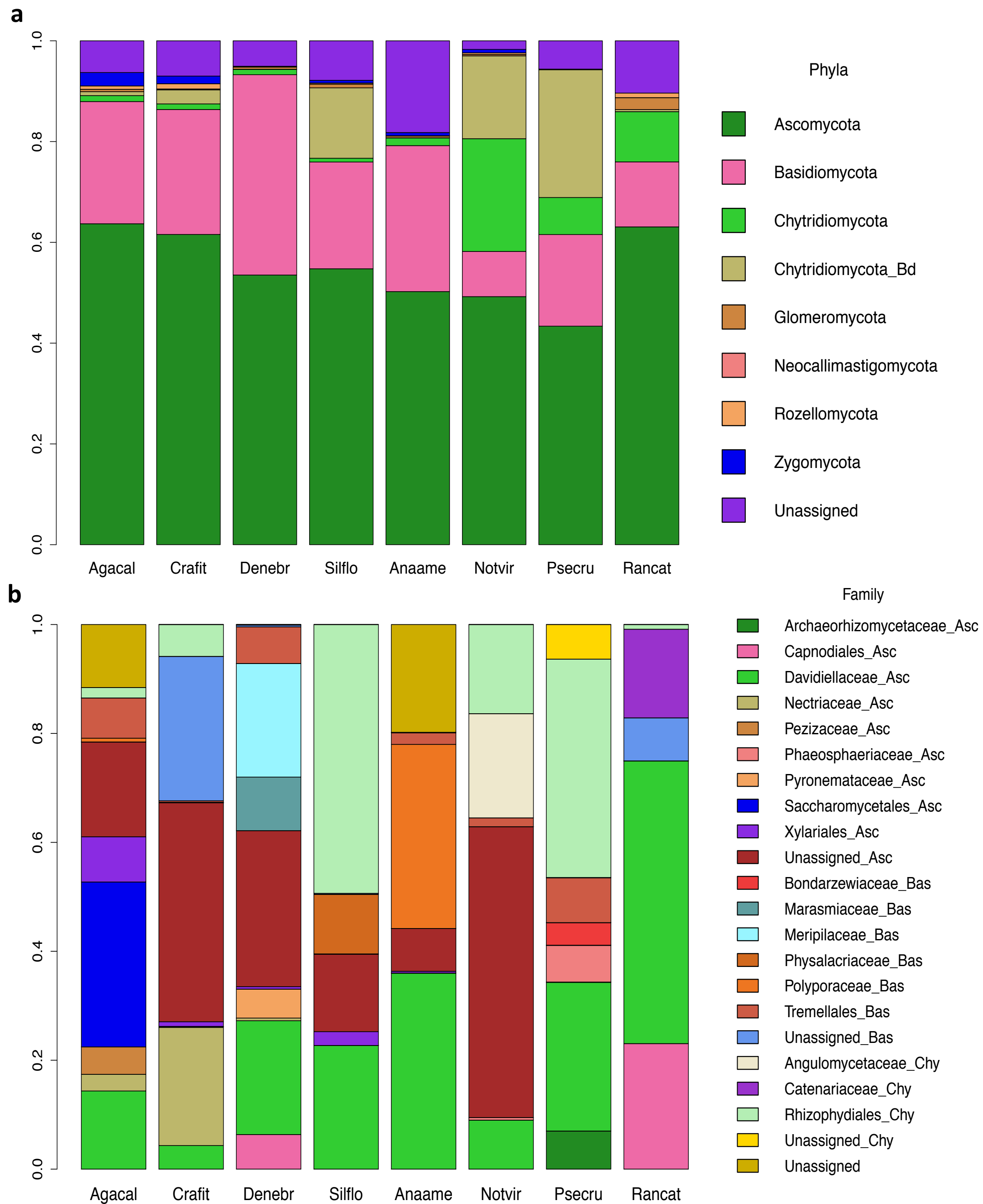

Figure 1. Stacked barplots showing the relative abundance of the fungal phyla (A) and orders/families (B) across amphibian host species. The taxonomic orders and families denoted in the figure correspond to abundant OTUs with a maximum relative abundance cut-off equal or above 0.20. Taxonomic orders represent OTUs with an undefined classification at the family level (*incertae sedis*) based on the database UNITE.

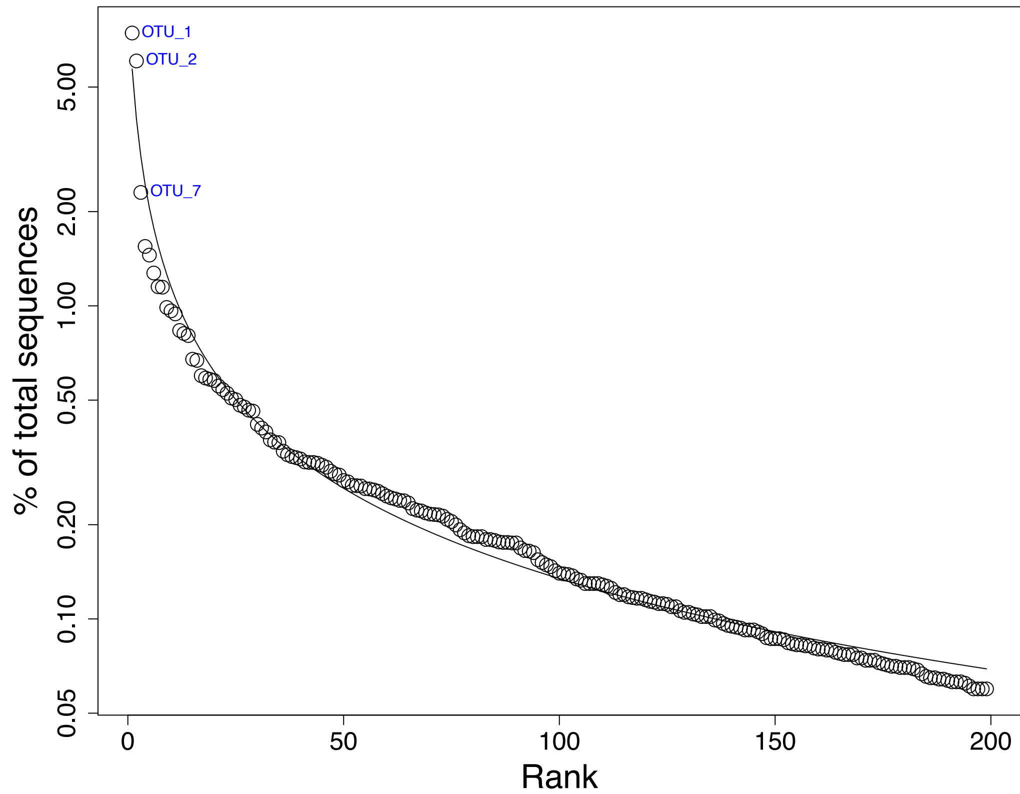

Figure 2. Diversity/dominance plot showing the rank order of the 200 most abundant OTUs based on the percentage (%) of each one out of the total number of sequences. In this analysis, the abundant OTUs were selected using a maximum relative abundance cut-off equal or above 0.05. OTU_1 (genus: *Cladosporium*) and OTU_2 represent OTUs from the phyla Ascomycota, and OTU_7 represents an OTU identified as Bd.

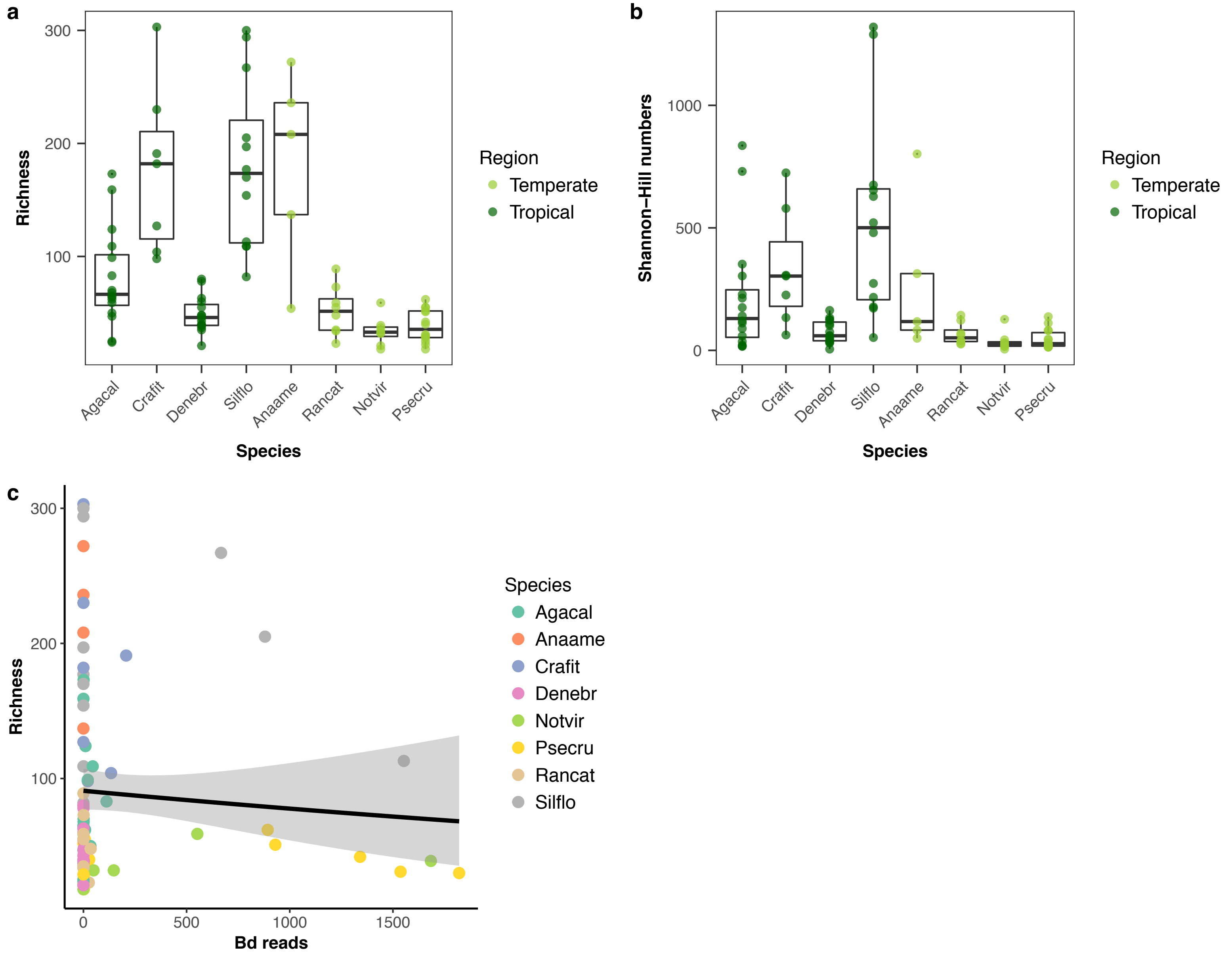

Figure 3. Alpha diversity values for OTU richness (A) and Shannon Index transformed to Hill numbers (B) across amphibian host species; and relationship between infection load (number of Bd reads) and richness based on a GLM (R^2 = 0.006, P = 0.42) and 95% CI (C). Points in figures represent the alpha diversity values of each individual amphibian. In figure C, the amphibian species are denoted by the first three letters of the genus and the first three letters of the species name. Agacal: *Agalychnis callidryas*; Crafit: *Craugastor fitzingeri*; Denebr: *Dendropsophus ebraccatus*; Silflo: *Silverstoneia flotator*; Anaame: *Anaxyrus americanus*; Notvir: *Notophthalmus viridescens*; Psecru: *Pseudacris crucifer*; Rantcat: *Rana catesbeiana*.

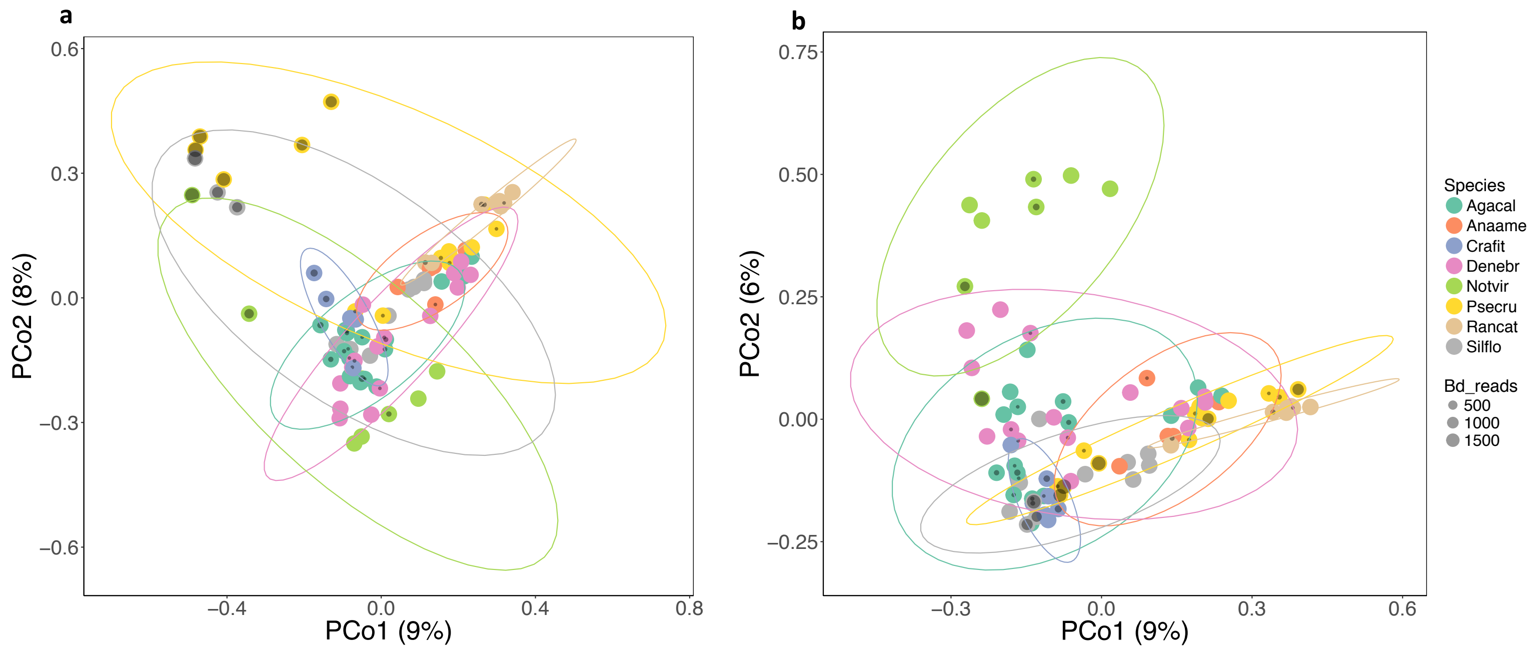

Fig. 4. Beta diversity of fungal communities across amphibian host species using a principal coordinate analysis (PCoA) ordination based on Bray-Curtis dissimilarity distances. The ordinations show the clustering pattern including (A) and excluding Bd OTUs (B) from the analysis. Points represent single individuals from the different amphibian species. Dark circles within points represent the number of Bd reads determined for the respective individual. Amphibian species are denoted by the first three letters of the genus and the first three letters of the species name. Agacal: *Agalychnis callidryas*; Crafit: *Craugastor fitzingeri*; Denebr: *Dendropsophus ebraccatus*; Silflo: *Silverstoneia flotator*; Anaame: *Anaxyrus americanus*; Notvir: *Notophthalmus viridescens*; Psecru: *Pseudacris crucifer*; Rancat: *Rana catesbeiana*.

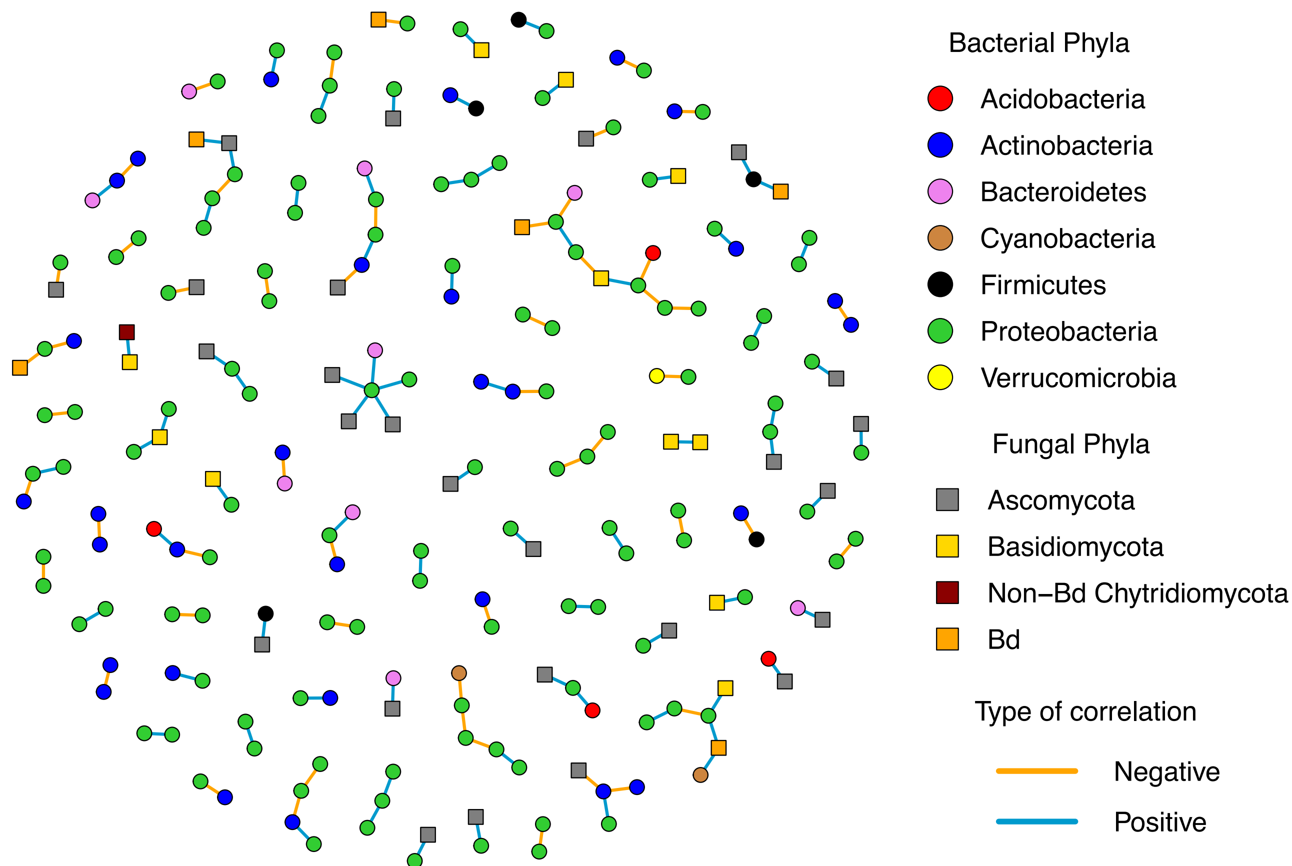

Fig. 5. Significant co-occurrences of fungal and bacterial OTUs from a subset of 63 amphibian skin samples for which both datasets were available. Nodes denote single OTUs, and edges represent significant (pseudo *P*-values < 0.05) correlations between OTUs based on SparCC default parameters. The figure include correlation with coefficients smaller than -0.35 and equal or larger than 0.35. Node shapes denote taxonomic Domain, and colors their respective phylum.

Supplementary material

Table S1 Results of *post hoc* comparisons of the skin fungal communities across amphibian host-species based on alpha diversity metrics: richness and Shannon index transformed to Hill numbers.

Pairwise comparisons		**Richness**			**Shannon index Hill numbers**		
		Std. Error	*z* value	Adjusted *P* value	Std. Error	*z* value	Adjusted *P* value
A. callidryas -	*A. americanus*	0.2044	4.001	**0.0015**	0.0017651	-0.553	0.99898
A. callidryas -	*C. fitzingeri*	0.1811	4.362	**<0.001**	0.0014254	-1.153	0.92306
A. callidryas -	*D. ebbracatus*	0.1476	-3.355	**0.0175**	0.0031478	2.693	0.09178
A. callidryas -	*N. viridescens*	0.1815	-4.718	**<0.001**	0.0085688	2.653	0.10233
A. callidryas -	*P. crucifer*	0.1516	-4.817	**<0.001**	0.0051057	3.261	**0.01711**
A. callidryas -	*R. catesbiana*	0.178	-2.425	0.2238	0.0047928	2.175	0.29824
A. callidryas -	*S. flotator*	0.153	5.347	**<0.001**	0.0011236	-2.476	0.15742
A. americanus -	*C. fitzingeri*	0.2324	-0.12	1	0.0017514	-0.381	0.99991
A. americanus -	*D. ebbracatus*	0.2074	-6.332	**<0.001**	0.0033082	2.858	0.05859
A. americanus -	*N. viridescens*	0.2328	-7.193	**<0.001**	0.008629	2.747	0.07941
A. americanus -	*P. crucifer*	0.2103	-7.363	**<0.001**	0.0052061	3.386	**0.01123**
A. americanus -	*R. catesbiana*	0.23	-5.432	**<0.001**	0.0048997	2.327	0.22007
A. americanus -	*S. flotator*	0.2113	0	1	0.0015159	-1.191	0.90969
C. fitzingeri -	*D. ebbracatus*	0.1845	-6.968	**<0.001**	0.0031401	3.223	**0.01949**
C. fitzingeri -	*N. viridescens*	0.2126	-7.745	**<0.001**	0.008566	2.845	0.06137
C. fitzingeri -	*P. crucifer*	0.1877	-8.1	**<0.001**	0.0051009	3.586	**0.00557**
C. fitzingeri -	*R. catesbiana*	0.2096	-5.829	**<0.001**	0.0047878	2.521	0.14134
C. fitzingeri -	*S. flotator*	0.1888	0.148	1	0.0011019	-1.033	0.95619
D. ebbracatus -	*N. viridescens*	0.1849	-1.955	0.5049	0.0090141	1.581	0.70057
D. ebbracatus -	*P. crucifer*	0.1556	-1.512	0.796	0.0058221	1.404	0.81108
D. ebbracatus -	*R. catesbiana*	0.1814	0.35	1	0.0055497	0.351	0.99995
D. ebbracatus -	*S. flotator*	0.1569	8.368	**<0.001**	0.0030151	-3.734	**0.00305**
N. viridescens -	*P. crucifer*	0.1881	0.671	0.9976	0.0098698	-0.616	0.99795
N. viridescens -	*R. catesbiana*	0.21	2.024	0.4578	0.0097116	-1.267	0.87913
N. viridescens -	*S. flotator*	0.1892	8.849	**<0.001**	0.008521	-2.994	**0.03924**
P. crucifer -	*R. catesbiana*	0.1847	1.617	0.7335	0.0068527	-0.908	0.97845
P. crucifer -	*S. flotator*	0.1607	9.633	**<0.001**	0.005025	-3.867	**0.00178**
R. catesbiana -	*S. flotator*	0.1858	6.725	**<0.001**	0.0047068	-2.806	0.06824

Chapter 5: Synthesis

Animals harbor diverse host-associated microbial communities that can play important roles in different aspects of an animal's life-history, including protection against pathogens, thus influencing overall host health and fitness (Hird, 2017; McFall-Ngai et al., 2013). However, despite evidence for a context-dependency in the outcome of symbiotic interactions (e.g. host-pathogen interactions), the influence of environmental conditions on the structure and function of host-associated microbial communities remains poorly understood. In Chapter 2, by conducting a field survey across low and high elevation sites to assess the skin bacterial communities and metabolite profiles associated with the amphibian species *Silverstoneia flotator*, I provided evidence that host-associated bacterial communities can be dynamic and that it is likely that their function on the host depends on the environmental conditions (Medina et al., 2017a). Overall, I determined that while the diversity and structure of the skin bacterial communities remained consistent across elevations and sites, there was variation in the metabolite profiles. These results suggested that similar communities, potentially mediated by differences in environmental conditions, produce different metabolites at different locations, implying a potential functional plasticity in these bacterial communities. Considering that Bd outbreaks have been more dramatic at high elevations compared to the lowlands, I believe that future research using a shotgun metagenomic or a transcriptomic approach are promising areas to elucidate the environmentally-induced changes in genetic pathways and expression of genes involved in host-microbe interactions and antifungal function (Rebollar et al., 2016). Lastly, I recommend that conservation efforts based on the use of probiotics to mitigate infections by Bd (Bletz et al., 2013) should consider the context-dependent function of probiotic candidates, in particular within a range of conditions similar to those at sites of future amphibian reintroductions.

Culturing isolates represents a critical step in characterizing the metabolic, pathogenic and functional features of the vast microbial diversity identified using culture-independent methods (Zengler et al., 2002). In the amphibian-skin microbiome study system, the isolation of amphibian skin bacteria into pure cultures has been a critical approach for determining the functional ability of these bacteria to inhibit Bd-growth in *in vitro* challenge assays, and to assess their potential use as probiotics for amphibian conservation (Bletz et al., 2013). However, across a number of studies, the cultivation and isolation of these amphibian skin bacteria has been restricted to mainly one culture media (R2A), despite little evidence of its superiority for isolating the majority of the skin bacteria. Thus, in Chapter 3, using skin swabs from toads (i.e. *Anaxyrus americanus*), I conducted an experiment to compare the diversity and community structure among the plated communities growing on distinct culture media that varied in nutrient concentration (high vs. low concentration). In general, I found that culture media with low nutrient concentrations (R2A and diluted R2A), relative to other common media with higher nutrient concentrations (LB and TSA), allowed the growth of a higher diversity of bacterial taxa and recovered a higher proportion of the diversity occurring in the amphibian skin, including the most abundant taxa (Medina et al., 2017b). This study corroborates that using R2A to culture amphibian skin bacteria is superior to other commonly used media. In addition, this study highlights the importance of considering microbial interactions, such as competitive-exclusion by fast-growing bacteria, and the nature of the culture media to improve our ability to culture rare or previously uncultured microbes. Lastly, considering that differences among toads influenced the structure of the plated bacterial communities more than the media types, I also

recommended that swabbing more individuals might be the best way to maximize culture collections, regardless of the type of culture media.

In the last decade, the study of bacterial communities associated with amphibian skin has substantially contributed to our understanding of the ecology of animal-associated microbial communities (Walke and Belden, 2016), and their potential use to control infections by emergent pathogens, such as Bd (Bletz et al., 2013). One of the major lessons learned from this research is that there are challenges to using bacteria as probiotics to mitigate Bd infections in endangered amphibians, among which is the failure of probiotic colonization of the skin following exposure (Becker et al., 2011; Küng et al., 2014). These failed trials have, in part, motivated recent studies assessing the fungal portion of the amphibian skin microbiome, which are still scarce and lagging behind those on bacteria. Fungi often interact with bacteria in natural systems, and non-Bd resident fungi could certainly play a role in these complex skin microbial communities. Thus, in Chapter 4, using previously isolated DNA from skin swabs from free-living amphibians, I assessed the fungal communities associated with the skin of eight host species from either a temperate or a tropical region, and evaluated the influence of infections by Bd on the diversity and structure of these skin fungal communities. In addition, I was interested in identifying potential interactions between the bacteria and fungi co-occurring in the amphibian skin. The results from this study suggested that, consistent with what is known for the amphibian skin bacteria, the fungal diversity varies across amphibian species, where terrestrial species harbor a higher richness, and that these communities are host specific in terms of community structure. Specifically, I determined that amphibian species identity was the most important predictor of the fungal community structure, followed by region. However, infections by Bd did not seem to influence the diversity and structure of the fungal communities. Moreover, I learned that fungi from the major fungal phyla (i.e. Ascomycota and Basidiomycota) dominated the skin fungal communities of the amphibian species used in my study, and that species from these fungal phyla might interact with co-occurring bacteria within the amphibian skin. Overall, I suggest that the feedbacks potentially induced by fungi in the amphibian skin microbiome adds another layer of complexity to studies focusing on the mechanisms shaping skin community assembly and overall function against Bd.

In conclusion, based on studying completely different aspects of the amphibian skin microbiome, I believe that host-associated microbial communities provide a useful model system to address broad ecological questions through either observational or experimental studies. For instance, using a combination of omics methods, host-associated microbial communities allow the assessment of the environmental context-dependency of the relationship between diversity and function, including function against invasive species, such as pathogens. In addition, host-associated microbial communities provide a suitable model system to assess the role of local interactions (i.e. within the host) in shaping diversity patterns at larger scales (i.e. host population and metapopulation). Moreover, considering the role of environmental transmission on diversity maintenance of host-associated microbial communities, research focus on metacommunity theory as a driver of diversity patterns will benefit from the study of host-associated microbial communities. Lastly, the high degree of experimental control offered by microbial systems allows the design of comprehensive manipulative experiments to address complex ecological questions difficult to address using other systems, such as plants and animals (reviewed in Jessup et al., 2004).

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