Geography, Host Genetics, and Cross-Domain Microbial Networks Structure the Skin Microbiome of Fragmented Brazilian Atlantic Forest Frog Populations

Anat Belasen[1], Maria Riolo[1], Mariana Lúcio Lyra[2], Felipe Toledo[3], and Tim James[1]

[1]University of Michigan
[2]Universidade Estadual Paulista Julio de Mesquita Filho - Campus de Rio Claro
[3]University of Campinas Institute of Biology

May 6, 2020

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412300 — This a preprint and has not been peer reviewed. Data may be preliminary.

Abstract

The host-associated microbiome plays a significant role in health. However, the roles of factors such as host genetics and microbial interactions in determining microbiome diversity remain unclear. We examined these factors using amplicon-based sequencing of 175 Thoropa taophora frog skin swabs collected from a naturally fragmented landscape in southeastern Brazil. Specifically, we examined (1) the effects of geography and host genetics on microbiome diversity and structure; (2) the structure of microbial eukaryotic and bacterial co-occurrence networks; and (3) co-occurrence between microeukaryotes with bacterial OTUs known to affect growth of the fungal frog pathogen Batrachochytrium dendrobatidis (including anti-Bd bacteria commonly referred to as "antifungal"). Microbiome structure correlated with geographic distance, and microbiome diversity varied with both overall host genetic diversity and diversity at the frog MHC IIB immunity locus. Our network analysis showed the highest connectivity when both eukaryotes and bacteria were included, implying that ecological interactions occur among Domains. Lastly, anti-Bd bacteria did not demonstrate broad negative co-occurrence with fungal OTUs in the microbiome, indicating that these bacteria are unlikely to be broadly antifungal. Our findings emphasize the importance of considering both Domains in microbiome research, and suggest that probiotic strategies for amphibian disease management should be considered with caution.

Introduction

The host-associated microbiome has recently captured the attention of researchers seeking to understand and predict disease-associated wildlife population declines. In particular, research on the skin microbiome is burgeoning in the field of amphibian disease, in which a majority of studies focus on the skin disease chytridiomycosis caused by the pathogenic chytrid fungus *Batrachochytrium dendrobatidis*(*Bd*). *Bd* and other pathogens have been linked to severe amphibian declines around the world since at least the 1970s (1–4). In some regions, however, declines have not been observed. Plethodontid salamander populations from the Eastern United States showed no evidence of disease-associated declines despite the presence of *Bd* in the environment (5). In a series of foundational studies, many of which were performed *in vitro* , bacteria cultured from salamander skin were correlated with reduced disease risk (6–8). Further studies pointed to antifungal bacterial metabolite production as the main mechanism behind this correlation between bacteria and reduced disease risk (7,9,10). These findings among others gave rise to interest in characterizing amphibian microbiome bacteria as a means to determine *Bd*susceptibility, and in using "probiotic strategies" (manipulating amphibian skin bacteria) to mitigate disease-associated amphibian declines (10–13).

However, despite a growing body of research on specific*Bd* -inhibitory bacteria, much remains to be understood about the diversity and assembly of the overall amphibian skin microbiome, including the ecological

roles of non-bacterial taxa (but see Kueneman et al. 2016; Kearns et al. 2017) and interactions between microbiome bacteria and microeukaryotes other than *Bd* . A diversity of microeukaryotes including fungi, microscopic metazoans, and protists have been identified on amphibian skin using high-throughput sequencing (16,17). In previous studies, fungi comprised the dominant eukaryotic taxon on adult amphibians (14), and explained more variation in*Bd* susceptibility than bacteria (15). Although little is known about the ecological roles of these fungi in the amphibian skin microbiome, symbiotic fungi are known to serve important roles in protection against fungal pathogens in other host-microbial systems (Gao et al. 2010; Newsham et al. 1995). Fungi also serve as hyperparasites,*i.e.* , parasites of pathogens/parasites. For example, the cryptomycete fungus *Rozella* parasitizes chytrid fungi (20). Less is known about the symbiotic roles of host-associated protists, although microbiome eukaryotes on the whole have been shown to impact health (21,22) and immune function (23) in mammals. Thus, microbiome eukaryotes could be equally important as bacteria in determining disease susceptibility in vertebrates including amphibians. Without an understanding of the interactions between microbiome eukaryotes and bacteria, it is impossible to predict the potential microbiome-wide effects of proposed measures to manipulate bacteria to control *Bd*outbreaks.

In addition, few studies to date have examined the genetic mechanisms that determine animal microbiome assembly and diversity. From research on mammals, it is known that microbiome assembly and diversity can co-vary with overall host genetic diversity (24) as well as host immunogenetics (25,26), with the latter relationship hypothetically resulting from interactions between immune cells and microbes including commensals and pathogens. In amphibians, previous studies have demonstrated that geography, host identity, and developmental stage can influence microbiome diversity (27–29). Yet only a single study to date has linked amphibian skin microbiome diversity with overall host genetic variability (29). Although no studies have directly examined the relationship between immunogenetics and microbiome diversity or structure in amphibians, results from an experimental study on the laboratory model frog *Xenopus laevis* suggest that MHC (major histocompatibility complex) immunogenes may determine the ability of hosts to tolerate different microbes (30). The relationship between immunogenes and the amphibian host-associated microbiome remains to be explored, and is increasingly relevant for wild amphibian populations threatened by emerging disease.

In a number of amphibian species, genetic diversity has been compromised due to anthropogenic habitat fragmentation (31). Although it is unknown to what extent habitat fragmentation impacts the amphibian skin microbiome, genetic erosion in fragmented amphibian populations has been observed at neutral loci as well as immunogenetic regions (17) which may have implications for microbiome structure (25). In addition, fragmentation may cause a decline in microbial transmission, which in turn may alter microbial interactions and networks in host-associated microbiomes. However, the effects of habitat fragmentation on wildlife are subject to time lags (32); genetic erosion resulting from inbreeding may not be detectable for several generations following habitat fragmentation, making the impacts on genetics and related factors difficult to detect in recently fragmented populations. Historically fragmented populations offer an opportunity to examine the effects of genetic erosion on the microbiome and broader animal health.

We evaluated the effects of long-term habitat fragmentation on the amphibian skin microbiome using a historically fragmented model system in the Brazilian Atlantic Forest. This system consists of dozens of land-bridge islands, which were naturally separated from the mainland 12,000-20,000 years ago via sea level rise (33) and thus represent ancient forest fragments. Contemporary insular frog populations were once part of contiguous coastal populations, and are now isolated to the islands (34,35). Using this geographic setting, we examined the impacts of geography and host genetics on skin microbiome diversity and structure. We used amplicon-based high-throughput DNA sequencing to analyze bacterial and eukaryotic microbes found in skin swab samples collected from a single frog species (*Thoropa taophora*[Cycloramphidae]) found across coastal mainland and island sites. The island populations of *T. taophora* have experienced fragmentation-induced genetic erosion at both neutral and immunogenetic loci (17,35), offering an opportunity to examine the relationship between host genetic diversity and microbiome diversity and structure. We compared the bacteria we recovered from *T. taophora* skin swabs to a database of amphibian microbiome bacterial isolates that have been previously categorized as *Bd* inhibitory, *Bd* enhancing, and having no effect on *Bd* . This

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412300 — This a preprint and has not been peer reviewed. Data may be preliminary.

allowed us to test for corresponding ecological relationships between these bacteria and other microeukaryotes found in the *T. taophora* microbiome. Our study was designed to address the following research questions: (1) Does geography and/or host genetic diversity structure the microbiome community? (2) How is bacterial diversity and community assembly related to microeukaryotic diversity and community assembly in the skin microbiome? (3) Do bacteria that affect *Bd* growth have predictable associations with other microbiome eukaryotes?

Methods

Study system and field sampling

The focal species for this study is *Thoropa taophora,* a cycloramphid frog with a unique tolerance for coastal habitat that allows a wide distribution across the coastal Atlantic Forest of São Paulo State (36). Adult *T. taophora* frogs (n=175 total) were sampled from each of ten study populations: seven island populations and three coastal mainland populations (Fig. 1, Table 1). Genetic diversity is lower in island *T. taophora* populations relative to coastal mainland populations, both at neutral (microsatellite) loci (35) as well as at the MHC IIB immunogenetic locus (17). To examine how host genetics impact skin microbiome diversity, skin swab samples were taken from the same individuals that were previously genotyped at MHC IIB (see Belasen et al. 2019). Frogs were thoroughly washed with sterile (autoclaved) distilled water and then swabbed on the ventral surface using standard protocols that minimize cross-contamination (37). DNA was extracted from swabs using a Qiagen DNeasy Blood and Tissue kit.

Microbiome Sequencing and Bioinformatic Processing

Individual swab DNA extracts were barcoded, pooled and sequenced on the Illumina MiSeq platform (250 bp paired-end reads) in two assays: (1) barcoded 16S primers (515F and 806R) (38) were used to examine bacterial diversity; and (2) barcoded 18S v4 primers (TAReuk454FWD1 and TAReukREV3) (39) were used to examine microeukaryote diversity. 16S libraries were constructed at the Universidade Estadual Paulista (BR) and sequenced at the Tufts University Core facility (USA) while 18S library preparation and sequencing was performed at the University of Michigan (USA). Negative (template-free) controls were run simultaneously with each sequencing library to ensure there was no contamination from PCR or sequencing reagents.

Sequences were quality-filtered and processed using the Quantitative Insights into Microbial Ecology (QIIME) MiSeq pipeline using default settings (40). As no mock community was included as a positive sequencing control, low abundance OTUs were filtered from the dataset using a conservative abundance threshold (<0.005% of all reads) (41). Sequences were clustered into operational taxonomic units (OTUs) using a 97% similarity threshold and compared against reference databases (rdp GreenGenes for 16S, Silva 97 for 18S) to assign taxonomy using the BLAST search algorithm. Chimeras were identified and filtered using UCHIME2 (42). For 18S data, sequences assigned to frog or other non-target non-microbial species (*e.g.* , Streptophyta) were filtered from the dataset. Sequences were rarefied to 1000 per sample for 18S data and 2000 per sample for 16S data. These values were selected based on visual examination of histograms and read accumulation curves constructed for all samples.

To infer ecological effects of bacteria on fungi and protists, bacterial OTU representative sequences from the *T. taophora* samples were compared against a reference database containing bacteria that were previously isolated from amphibian skin and evaluated for effects on*Bd* growth in co-culture experiments (43). The BLAST algorithm was implemented and an E-value threshold of E < 1e-20 was used to identify OTU matches with the reference database. Matching *T. taophora* skin bacteria were categorized as *Bd* enhancing,*Bd* inhibiting, or having no effect on *Bd* growth.

Data Analysis

To evaluate overall patterns of microbiome alpha diversity, t-tests and Mann-Whitney U tests were performed to compare total observed eukaryotic or bacterial OTUs across site types (island vs. mainland) or MHC IIB genotypes (homozygote vs. heterozygote) in SPSS (vrs. 22). Analyses of microbiome community structure

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412300 — This a preprint and has not been peer reviewed. Data may be preliminary.

(beta diversity) were conducted using Mantel tests of community dissimilarity vs. geographic distance or genetic distance (F_{ST}) implemented in the ade4 package of R (vrs. 1.7-11) (44–47).

To examine associations between microbial communities and geography or host frog MHC IIB genotype, data were statistically analyzed and visualized using packages implemented in Python (vrs. 2.7.13) and Matplotlib (48,49). Associations between microbial communities and geography or frog MHC IIB genotype were determined by simulating an expected null distribution of host frog microbiomes. To create the null distribution, a two-column data table was first created with column 1 being the site type (island or coastal) or MHC IIB genotype (heterozygous or homozygous) of a host frog and column 2 being one microbial OTU found on that frog. After the data table was populated for all frogs and microbes in the dataset, column 2 (microbial OTU) was held constant while column 1 (site type or frog genotype) was shuffled randomly. This was repeated 1000 times to create two sets of random microbial occurrence distributions, one for analysis of microbial associations with site type and a second for analysis of microbial associations with host frog genotype.

Co-occurrence between microbial OTUs within and among domains (Bacteria vs. Eukaryotes) was analyzed with a third null distribution of microbial communities. Because of potential site effects on microbial presence and community structure (*e.g* ., some microbes only co-occur on frogs because the microbes themselves solely occur at the same subset of sites) and site-MHC IIB genotype interactions (as homozygotes and heterozygotes are not evenly distributed across sites or site types; Table 1), an expected null distribution of microbes accounting for site-specific presence/absence of each microbe was created. This null distribution of microbes was achieved through within-site randomization using MCMC edge swapping, a standard method for network datasets (50–52). This method allows any configuration to be reached from any starting point, and allows for even sampling along all allowed states as forward and backward swaps are equally likely. To achieve this, first, two microbe-frog pairs were randomly selected (each pair consisting of a single randomly selected microbial OTU found on a single randomly selected frog). Microbial OTUs were then swapped between the selected frogs when three criteria were met: (1) the frogs were different individuals with the same MHC IIB genotype (either both homozygous or both heterozygous); (2) the OTUs were different from one another; and (3) neither frog already hosted the microbe it would receive via the swap. Microbe swapping was performed with 1000 repetitions for each frog-microbe pair.

To test whether hypothesized bacterial effects on *Bd* extend to diverse microeukaryotic members of the microbiome, bacterial OTUs that matched the Woodhams et al. (2015) database were binned according to their hypothesized ecological significance with regard to *Bd*(*Bd* inhibitory, *Bd* enhancing, or no effect on *Bd*). The co-occurrences of bacteria within each category with microbiome eukaryotes were then compared with the third null distribution of microbial OTUs.

For all microbial association/co-occurrence analyses, the probability of non-random microbial association/co-occurrence (p) was calculated by comparing observed versus expected counts of microbial association/co-occurrence. P -values were evaluated at a significance level of $\alpha = 0.05$. Using the results of the tests of co-occurrences within and among all microbial taxa, network analyses were performed and visualized in SciPy (53).

Results

Associations between geography, host genetics, and the skin microbiome

There were 845 microeukaryotic OTUs and 303 bacterial OTUs recovered across all samples after filtering and rarefaction. Microeukaryotic diversity was positively correlated with bacterial diversity across all samples (number of OTUs; Spearman's $\rho = 0.25$, $p < 0.001$). Mantel tests revealed significant positive associations between geographic distance and beta diversity in both eukaryotic and bacterial microbes: populations that were geographically closer showed significantly more similar microbiome community structure (eukaryotic taxa: $r = 0.18$, $p < 0.05$; bacterial taxa: $r = 0.40$, $p < 0.01$, Fig. 2). Neither MHC IIB genetic distance nor neutral genetic distance (from microsatellite data published in Duryea et al. 2015) were associated with microbiome community structure ($p > 0.1$ for all Mantel tests between 18S or 16S beta diversity and F_{ST}

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412300 — This a preprint and has not been peer reviewed. Data may be preliminary.

matrices for MHC IIB or microsatellites).

In the 16S bacterial dataset, Proteobacteria were dominant across all samples, both by number of OTUs and sequence reads (Fig. 3A&B). Proteobacteria also formed the core bacterial microbiome across samples (Fig. 3C). Among the eukaryotic microbiota, fungi were dominant by both number of OTUs and sequence reads (Fig. 3D&E). No core group of eukaryotic taxa was recovered, though some fungal OTUs were found in approximately 50% of samples (Fig. 3F). These common fungal OTUs included Ascomycota, Basidiomycota, and unidentified fungi.

Bacteria diversity was similar across site types (Mann-Whitney tests, $p > 0.05$), but island frogs had fewer eukaryotic OTUs than mainland frogs (85.5 OTUs on average on islands vs. 110.5 on average on the mainland; Mann-Whitney test, $U = 2{,}604$, $p = 0.001$). However, site type was associated with variation in composition of both bacteria and microeukaryotes (Fig. 4A&B). Eight bacterial groups were significantly associated with site type: Cyanobacteria and Proteobacteria were statistically associated with coastal mainland sites, while six bacterial groups were statistically associated with island sites. Among the microeukaryotes, Rhizaria, Nucleariids, Ichthyosporeans, and Apusozoans were statistically associated with coastal sites while Fungi and Apicomplexans were statistically associated with island sites.

While the number of bacterial OTUs was not significantly different between MHC IIB homozygotes and heterozygotes (t-test, $p > 0.05$), MHC IIB homozygotes possessed fewer microeukaryote OTUs than MHC IIB heterozygotes (16.6 on average on homozygotes vs. 29.7 on heterozygotes; Mann-Whitney U, $U = 3{,}729.5$, $p < 0.01$). However, this result could be confounded by differences across island and mainland sites: both microbial community composition (Fig. 4A&B) and the number of MHC IIB heterozygotes and homozygotes (Table 1) vary across site types. Therefore, the analysis of microeukaryote diversity against MHC genotypes was repeated on a subset of the data only including individuals from mainland sites. MHC IIB homozygotes possessed significantly fewer microeukaryote OTUs than heterozygotes when only mainland frogs were included in the analysis (19.2 on average on homozygotes vs. 39.1 on heterozygotes; Mann-Whitney U, $U = 681.5$, $p < 0.01$).

Microbiome community composition varied between MHC IIB heterozygotes and homozygotes for both bacteria and microeukaryotes when compared with null expectations based on genotype randomizations. MHC IIB heterozygotes hosted significantly more unidentified Bacteria, Bacteroidetes, and Firmicutes, but fewer Actinobacteria and Proteobacteria OTUs than homozygotes (Fig. 4C). In terms of microeukaryotes, MHC IIB heterozygotes hosted significantly more OTUs belonging to the Ciliates, Rhizaria, and Stramenopiles, but significantly fewer Fungi and Algae OTUs than homozygotes (Fig. 4D).

Microbial networks within and among domains

Separate networks were constructed for bacteria and microeukaryotes based on tests of co-occurrence between OTUs within and among taxonomic groups within Domains (Fig. S1). Network connections indicate that taxa co-occur more frequently than expected by random chance. A dominant bacterial network assembled that consisted of Firmicutes and Bacteroidetes at the center with connections to Fusobacteria, Spirochaetes, Verrucomicrobia, Deferribacteres, and unidentified bacteria (Fig. S2). A second group was composed of Gemmatimonadetes and Cyanobacteria. Actinobacteria and Proteobacteria did not form network connections with any other groups, although strong connections formed among OTUs within the Proteobacteria. Within the microeukaryotes, only one small network formed that consisted of five taxonomic groups: Non-apicomplexan Alveolates were at the center of the network and formed connections with Apicomplexans, Rhizaria, and unidentified microeukaryotes, which in turn connected with Nucleariids (Fig. S3). The remaining 16 microeukaryote taxa remained unconnected to the network, though there were strong connections among OTUs within the Algae.

The construction of a cross-Domain network revealed a greater number of connections among groups than either the bacterial or microeukaryotic network (Fig. 5). A majority of taxa (12/18) that had formed no connections in the bacteria-only and microeukaryote-only networks formed connections with other taxa in the overall microbial network. Specifically, these newly connected taxa included the two previously uncon-

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412300 — This a preprint and has not been peer reviewed. Data may be preliminary.

nected bacterial groups, Actinobacteria and Proteobacteria, and 10/16 previously unconnected microeukaryote groups.

Associations between microbiome eukaryotes and bacteria reported to inhibit, enhance, or have no effect on Bd *growth*

When *T. taophora* skin bacterial OTU representative sequences were compared against a published FASTA of bacterial OTUs that were previously isolated from a diversity of live amphibians and tested against *Bd* in co-culture inhibition experiments (Woodhams et al., 2015) nearly half (45%) of bacterial OTUs matched OTUs in the database (Fig. S4). Tests of co-occurrence between eukaryote groups and these matched bacterial OTUs revealed that enhancing, inhibitory, and no effect do not generally reflect the associations of these bacteria with microeukaryotes generally or fungi specifically (Fig. 6). *Bd*enhancing bacteria were negatively associated with the Ascomycota and Basidiomycota fungi, and also showed strong but marginally non-significant positive associations with the Chytridiomycota and Choanoflagellates. *Bd* inhibitory bacteria showed significant positive associations with the Cryptomycota fungi and Choanoflagellates, and significant negative associations with the Basidiomycota fungi and other unidentified fungi. Finally, bacteria that were previously found to have no effect on *Bd* were positively associated with the Ascomycota, Choanoflagellates, and Rhizaria, and showed strongly but marginally non-significant positive associations with Algae, Amoebae, Ciliates, and Ichthyosporeans.

Discussion

Amphibian skin microbiomes exhibited high microeukaryote diversity and were dominated by Proteobacteria

In this study, we examined amphibian skin microbiome structure and diversity with respect to geography and host genetics. In analyzing both bacterial and microeukaryote sequences, we recovered microbial associations with geographic and host genetic factors, as well as unexpected patterns of microbial co-occurrence across domains. The diversity of microeukaryotes we recovered is higher than that previously reported from other wild frogs: we recovered 845 OTUs in our study compared with *e.g.* , 255 OTUs on *Rana cascadae* (16). In contrast, the level of bacterial diversity we recovered is lower than has been previously reported: in our study we recovered 303 bacterial OTUs compared with ˜600 OTUs on *Rana italica* in Federici et al. 2015). However, our recovery of bacteria from 11 phyla is within the range of taxonomic diversity previously recovered from amphibian skin, with for example 10-18 bacterial phyla reported from three species (55). Our analysis showed that total microeukaryotic and bacterial diversity were positively correlated across all samples, which is a novel finding to our knowledge. It seems unlikely that this pattern is an artifact of sequencing: different MiSeq runs (and different research facilities) were used to sequence microeukaryotes and bacteria.

Proteobacteria were the most dominant bacterial phylum on *T. taophora* skin across all study populations, in terms of both OTUs and reads (Fig. 3). This is similar to findings from bacterial microbiome studies of other tropical post-metamorphic anurans (56–59). One hypothesis for the dominance of Proteobacteria on frog skin is that many members of the Proteobacteria produce anti-*Bd* metabolites (60,61). The presence of a high number of Proteobacteria on *T. taophora* skin could hypothetically contribute to the low apparent susceptibility to *Bd* previously observed in this species (17). It is important to note however that the present study is correlative; without experimental manipulations it is difficult to pinpoint which factors (*e.g.* , the physiology of the skin, mucosal biochemistry, host-microbial evolutionary processes, or interactions with the saline coastal environment) are responsible for the overwhelming dominance of Proteobacteria on anuran skin.

Although bacteria were less diverse than microeukaryotes in our samples, bacteria could nevertheless dominate the skin microbiome according to microbial biomass, which we did not quantify in our study. Sequence reads are sometimes used as a proxy for relative abundance, but this has been shown to be an unreliable measure due to known sequencing biases among microbial taxa (62). It is possible that taxa representing fewer OTUs (*i.e.* , bacteria) represent a higher proportion of microbial biomass, and this should be considered in interpretations of our results. Future research to address the relationship between microbial diversity

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412300 — This a preprint and has not been peer reviewed. Data may be preliminary.

and abundance could utilize high-throughput sequencing alongside quantitative analyses, for example quantitative PCR.

Microbiome structure varied with geography and host immunogenetics

Geography was a significant factor in microbiome structure (beta diversity) for both bacterial and eukaryotic microbes. However, microbiome structure was not associated with genetic structure of populations at either neutral genetic markers or the MHC IIB immunogenetic locus. These results differ from a previous study on the frog *Amietia hymenopus,* which showed opposite patterns: there were no geographic effects on amphibian skin microbiome structure, but there was a significant association with population genetic structure (29). One possible explanation for the discrepancy between our results and the results from *A. hymenopus* (barring host identity factors) is that geographic structure in the host-associated microbial community is scale-dependent: our study spans a larger geographic area (˜100 km compared with ˜4 km in Griffiths et al. 2018). In addition, our study populations represent a set of connected mainland populations contrasted with a set of island populations that have been isolated for 12,000-20,000 years. The lack of association with genetic differentiation in our populations may be due to this relatively long period of divergence, or to isolation between island sites resulting in different environmental availability of microbes.

Microeukaryote diversity was associated with host genetic diversity, with genetically impoverished island populations possessing lower microeukaryotic diversity relative to coastal mainland populations (85.5 average OTUs on islands vs. 110.5 in coastal sites). This difference in microbiome diversity could be due to a number of factors, including less favorable environments or lower rates of host contact (*i.e.* , microbial transmission) on islands compared with coastal sites. However, MHC IIB heterozygosity was positively associated with microeukaryotic diversity even when only coastal populations were analyzed (19.2 average OTUs on coastal homozygotes vs. 39.1 on coastal heterozygotes). Taken together, these results imply that genetic diversity and/or MHC IIB genotype plays a significant role in determining microbiome diversity.

Microbiome structure also varied across site types and MHC IIB genotypes. Variation in microbiome structure among site types could be parsimoniously explained by variation in environmental filtering in coastal vs. island sites. However, these differences may also be driven by island isolation favoring longer-dispersing microbes, or alternatively by host genetic factors. The variation in microbiome structure across MHC IIB genotypes, although weaker than the variation due to site type, may be a clearer example of associations between endogenous host factors and the microbiome. Although MHC genes are thought to be primarily involved in pathogen resistance, results from laboratory and field studies suggest that MHC genotype and allelic composition can impact amphibian host-associated microbial assemblages (30,63). Together with our finding that microbiome structure and diversity are influenced by MHC genotype, this suggests that immune mechanisms conferred by MHC genes may influence the assembly of the overall microbiome.

Cross-Domain co-occurrence in the amphibian skin microbiome network

Our microbiome network analyses revealed a number of notable patterns. The bacterial network consisted of a major and minor group, and the majority of microeukaryote groups did not form significant connections in the eukaryote-only microbial network. However, in the overall microbial network, a number of microbial groups exhibited cross-Domain co-occurrence: a majority of previously unconnected microeukaryote groups (10/16) and both previously unconnected bacterial groups became connected in the overall microbiome network. To our knowledge, ours is the first study to demonstrate these cross-Domain network connections in the amphibian skin microbiome.

One important implication of this result is that ecological interactions may exist between microbiome bacteria and eukaryotes that may significantly impact microbiome assembly. It is currently unclear how widespread these associations are, as previous studies that have examined both bacteria and microeukaryotes on amphibian skin have focused on taxon-specific associations, namely between*Bd* -inhibitory bacteria and fungi (14), and between *Bd* and either bacteria or microeukaryotes (16). While potential antagonistic interactions with *Bd* have been the focus in cross-Domain research on the amphibian skin microbiome, microbial interactions can occur across the spectrum of biological symbioses (reviewed in 64). Mutualistic interactions

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412300 — This a preprint and has not been peer reviewed. Data may be preliminary.

between bacteria and microeukaryotes have been documented in other systems, for example mycorrhizae-helper bacteria are known to indirectly facilitate plant-fungal interactions in the multitrophic mycorrhizal complex (65). An alternative explanation for our network analysis results is that bacteria and eukaryotes positively co-occur due to co-filtering via specific host, environmental, or other exogenous factors unrelated to microbial interactions. Further research is needed on cross-Domain microbial co-occurrence patterns, microbial interactions and their implications for amphibian host health.

Bd inhibitory and enhancing bacteria have variable effects on microbiome fungi and protists

Our dataset included a number of bacteria previously shown to inhibit *Bd* , which have been generally termed "antifungal" in the literature (38) although empirical support for this broad designation comes from only a single study (14). Bacteria with previously demonstrated effects on *Bd* growth did not show general patterns with *T. taophora* skin microbiome eukaryotes. Bacteria previously found to enhance *Bd* growth were positively associated with the Chytridiomycota more broadly, although *Bd* was not present in our 18S dataset. However, these bacteria were negatively associated with Ascomycota and Basidiomycota fungi. Perhaps more critical are the relationships with *Bd* inhibitory bacteria, as these bacteria have been proposed for use in probiotic treatments for the management of *Bd* infections (11,66). *Bd* inhibitory bacteria showed weak positive associations with Cryptomycota fungi and significant negative associations with Basidiomycota fungi and other unidentified fungi in the *T. taophora* skin microbiome. *Bd* -inhibitory bacteria were also positively associated with Choanoflagellates, and showed strong though non-significant positive associations with the Zoopagomycota and Ichthyosporea.

Our results suggest that probiotic treatments in wild populations may have unintended consequences for microbiome stability. According to our analyses, specific attempts to increase *Bd* inhibiting bacteria and/or reduce *Bd* enhancing bacteria in wild frog populations could have unwanted effects, such as potentially reducing fungi in the Dikarya (Ascomycota and Basidiomycota) that are known to benefit amphibian health (Kearns et al. 2017), and/or augmenting poorly studied parasites such as Ichthyosporea protists (67) and fungi including Ascomycota and Zoopagomycota (68,69). These hypothetical effects warrant further study, for example through culture-based or *in vivo*challenges between proposed probiotic bacteria and these potentially impacted microeukaryotes.

Limitations and future research priorities

Taken together with recent studies (15,16), our results suggest that focusing only on bacteria provides an incomplete picture of the host-associated microbiome. Granted, as in many other amphibian microbiome studies (14,38) our study presents microbes at a relatively coarse phylogenetic resolution (generally phylum level). Very large differences in ecology and environmental requirements likely exist between OTUs within these higher-order classification levels, thus the patterns we detected may change with higher-resolution taxonomic data. With advancing technology allowing for increased sequence length (*e.g.* , third-generation sequencing), more efficient microbiome analysis pipelines, and well-curated reference sequence databases, future cross-Domain microbiome research at higher taxonomic resolution should be prioritized.

Our results imply that host immunogenes play a role in structuring the amphibian skin microbiome. Furthermore, our network analyses suggest that there may be important interactions between bacteria and microeukaryotes that have been missed by previous microbiome studies focusing on only one microbial Domain. Given the widespread use of bacterial probiotic treatments in humans as well as in domesticated and wild animals (70–72) and the interest in expanding these strategies to wild amphibians (66), future studies should prioritize advancing our understanding of interactions between microbiome bacteria and eukaryotes.

Acknowledgments

We thank Paula Morão and Luis Moreno for assistance in field logistics, field work, and labwork; Vinicius Hansser, Amanda Piffer, and Cesar Alexandre for assistance in the field; Carlos Almeida for providing site coordinates; Molly Bletz, Alisha Quandt, and William Davis for assistance with bioinformatics and data analysis; and Kelly Zamudio and Celio Haddad for assistance with fieldwork planning. We also thank

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412300 — This a preprint and has not been peer reviewed. Data may be preliminary.

members of the James Lab at the University of Michigan, Sabah Ul-Hasan, and Meg Duffy for valuable comments on early versions of the manuscript. We wish to acknowledge funding from NSF (OISE-1159513), CNPq (300980/2014-0), and a Block Grant from the U. Michigan Dept. of Ecology and Evolutionary Biology.

References

1. Lips K, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, et al. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proc Natl Acad Sci. 2006;103(9):3165–70.

2. Olson DH, Aanensen DM, Ronnenberg KL, Powell CI, Walker SF, Bielby J, et al. Mapping the Global Emergence of *Batrachochytrium dendrobatidis* , the Amphibian Chytrid Fungus. PLoS One. 2013;8(2):747–9.

3. Carvalho T, Becker CG, Toledo LF. Historical amphibian declines and extinctions in Brazil linked to chytridiomycosis. Proc R Soc B Biol Sci. 2017;284(1848).

4. Scheele BC, Pasmans F, Skerratt LF, Berger L, Martel A, Beukema W, et al. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. Science. 2019;363(6434):1459–63.

5. Muletz C, Caruso NM, Fleischer RC, McDiarmid RW, Lips KR. Unexpected rarity of the pathogen *Batrachochytrium dendrobatidis* in Appalachian Plethodon salamanders: 1957-2011. PLoS One. 2014;9(8):103728.

6. Harris RN, James TY, Lauer A, Simon MA, Patel A. Amphibian pathogen*Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. Ecohealth. 2006;3(1):53–6.

7. Harris RN, Lauer A, Simon MA, Banning JL, Alford RA. Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. Dis Aquat Organ. 2009;83(1):11–6.

8. Muletz CR, Myers JM, Domangue RJ, Herrick JB, Harris RN. Soil bioaugmentation with amphibian cutaneous bacteria protects amphibian hosts from infection by *Batrachochytrium dendrobatidis* . Biol Conserv. 2012;152:119–26.

9. Myers JM, Ramsey JP, Blackman AL, Nichols AE, Minbiole KPC, Harris RN. Synergistic inhibition of the lethal fungal pathogen*Batrachochytrium dendrobatidis* : The combined effect of symbiotic bacterial metabolites and antimicrobial peptides of the frog *Rana muscosa* . J Chem Ecol. 2012;38(8):958–65.

10. Woodhams DC, Brandt H, Baumgartner S, Kielgast J, Küpfer E, Tobler U, et al. Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. PLoS One. 2014;9(4).

11. Bletz MC, Loudon AH, Becker MH, Bell SC, Woodhams DC, Minbiole KPC, et al. Mitigating amphibian chytridiomycosis with bioaugmentation: Characteristics of effective probiotics and strategies for their selection and use. Ecol Lett. 2013;16(6):807–20.

12. Piovia-Scott J, Rejmanek D, Woodhams DC, Worth SJ, Kenny H, McKenzie V, et al. Greater Species Richness of Bacterial Skin Symbionts Better Suppresses the Amphibian Fungal Pathogen *Batrachochytrium dendrobatidis* . Microb Ecol. 2017;74(1):217–26.

13. Voyles J, Woodhams DC, Saenz V, Byrne AQ, Perez R, Rios-Sotelo G, et al. Shifts in disease dynamics in a tropical amphibian assemblage are not due to pathogen attenuation. Science. 2018;359(6383):1517–9.

14. Kueneman JG, Woodhams DC, Van Treuren W, Archer HM, Knight R, McKenzie VJ. Inhibitory bacteria reduce fungi on early life stages of endangered Colorado boreal toads (*Anaxyrus boreas*). ISME J. 2016;10(4):934–44.

15. Kearns PJ, Fischer S, Fernández-Beaskoetxea S, Gabor CR, Bosch J, Bowen JL, et al. Fight fungi with fungi: Antifungal properties of the amphibian mycobiome. Front Microbiol. 2017;8:1–12.

16. Kueneman JG, Weiss S, McKenzie VJ. Composition of micro-eukaryotes on the skin of the cascades frog (*Rana cascadae*) and patterns of correlation between skin microbes and *Batrachochytrium dendrobatidis* . Front Microbiol. 2017;8:1–10.

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412300 — This a preprint and has not been peer reviewed. Data may be preliminary.

17. Belasen AM, Bletz MC, Leite D da S, Toledo LF, James TY. Long-Term Habitat Fragmentation Is Associated With Reduced MHC IIB Diversity and Increased Infections in Amphibian Hosts. Front Ecol Evol. 2019;6:1–12.

18. Gao F-K, Dai C-C, Liu X-Z. Mechanisms of fungal endophytes in plant protection against pathogens. African J Microbiol Res. 2010;4(13):1346–51.

19. Newsham KK, Fitter AH, Watkinson AR. Arbuscular Mycorrhiza Protect an Annual Grass from Root Pathogenic Fungi in the Field. J Ecol. 1995;83(6):991.

20. Gleason FH, Carney LT, Lilje O, Glockling SL. Ecological potentials of species of *Rozella* (Cryptomycota). Fungal Ecol. 2012;5(6):651–6.

21. Hoffmann AR, Patterson AP, Diesel A, Lawhon SD, Ly HJ, Stephenson CE, et al. The skin microbiome in healthy and allergic dogs. PLoS One. 2014;9(1).

22. Holler E, Butzhammer P, Schmid K, Hundsrucker C, Koestler J, Peter K, et al. Metagenomic Analysis of the Stool Microbiome in Patients Receiving Allogeneic Stem Cell Transplantation: Loss of Diversity Is Associated with Use of Systemic Antibiotics and More Pronounced in Gastrointestinal Graft-versus-Host Disease. Biol Blood Marrow Transplant. 2014;20(5):640–5.

23. Graham AL. Ecological rules governing helminth microparasite coinfection. Proc Natl Acad Sci. 2008;105(2):566–70.

24. Benson AK, Kelly SA, Legge R, Ma F, Low SJ, Kim J, et al. Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc Natl Acad Sci. 2010;107(44):18933–8.

25. Blekhman R, Goodrich JK, Huang K, Sun Q, Bukowski R, Bell JT, et al. Host genetic variation impacts microbiome composition across human body sites. Genome Biol. 2015;16(1).

26. Marietta E, Rishi A, Taneja V. Immunogenetic control of the intestinal microbiota. Immunology. 2015;145(3):313–22.

27. Kueneman JG, Parfrey LW, Woodhams DC, Archer HM, Knight R, McKenzie VJ. The amphibian skin-associated microbiome across species, space and life history stages. Mol Ecol. 2014;23(6):1238–50.

28. Walke JB, Becker MH, Loftus SC, House LL, Cormier G, Jensen R V, et al. Amphibian skin may select for rare environmental microbes. ISME J. 2014;8(11):1–11.

29. Griffiths SM, Harrison XA, Weldon C, Wood MD, Pretorius A, Hopkins K, et al. Genetic variability and ontogeny predict microbiome structure in a disease-challenged montane amphibian. ISME J. 2018;1–12.

30. Barribeau SM, Villinger J, Waldman B. Ecological immunogenetics of life-history traits in a model amphibian. Biol Lett. 2012;8(3):405–7.

31. Allentoft ME, O'Brien J. Global amphibian declines, loss of genetic diversity and fitness: A review. Diversity. 2010;2(1):47–71.

32. Tilman D, May RM, Lehman CL, Nowak MA. Habitat destruction and the extinction debt. Nature. 1994;371(6492):65–6.

33. Suguio K, Angulo RJ, Carvalho AM, Corrêa ICS, Tomazeli LJ, Willwock JA, et al. Paleoníveis do mar e paleolinhas da costa. In: Quaternário do Brasil. 2005. p. 378.

34. Bell RC, Brasileiro CA, Haddad CFB, Zamudio KR. Evolutionary history of *Scinax* treefrogs on land-bridge islands in south-eastern Brazil. J Biogeogr. 2012;39(9):1733–42.

35. Duryea MC, Zamudio KR, Brasileiro CA. Vicariance and marine migration in continental island populations of a frog endemic to the Atlantic Coastal forest. Heredity. 2015;115(3):225–34.

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412300 — This a preprint and has not been peer reviewed. Data may be preliminary.

36. Duryea MC, Zamudio KR, Brasileiro CA. Characterization of microsatellite markers for Thoropa taophora (Anura, Cycloramphidae), a frog endemic to the Brazilian Atlantic Rain Forest. Mol Ecol Resour. 2008;8(3):663–5.

37. Hyatt AD, Boyle DG, Olsen V, Boyle DB, Berger L, Obendorf D, et al. Diagnostic assays and sampling protocols for the detection of*Batrachochytrium dendrobatidis* . Dis Aquat Organ. 2007;73(3):175–92.

38. Vences M, Lyra ML, Kueneman JG, Bletz MC, Archer HM, Canitz J, et al. Gut bacterial communities across tadpole ecomorphs in two diverse tropical anuran faunas. Sci Nat. 2016;103(3).

39. Stoeck T, Bass D, Nebel M, Christen R, Jones MDM, Breiner HW, et al. Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. Mol Ecol. 2010;19(SUPPL. 1):21–31.

40. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(510).

41. Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, et al. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat Methods. 2013;10(1):57–9.

42. Edgar RC. UCHIME2: improved chimera prediction for amplicon sequencing. 2016;

43. Woodhams DC, Alford RA, Antwis RE, Archer H, Becker MH, Belden LK, et al. Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. Ecology. 2015;96(2):595–595.

44. Dray S, Dufour AB. The ade4 package: implementing the duality diagram for ecologists. J Stat Softw. 2007;22(4):1–20.

45. Chessel D, Dufour AB, Thioulouse J. The ade4 package-I- One-table methods. R News. 2004;4:5–10.

46. Dray S, Dufour AB, Chessel D. The ade4 package-II: Two-table and K-table methods. R News. 2007;7(2):47–52.

47. R Core Team. R: A language and environment for statistical computing. R Found Stat Comput Vienna, Austria. 2018;

48. Hunter JD. Matplotlib: A 2D Graphics Environment. Comput Sci Eng. 2007;9:90–5.

49. van Rossum G. Python Tutorial, Technical Report CS-R9526. 1995.

50. Petersen J. Die Theorie der regulären graphs. Acta Math. 1891;15(1):193–220.

51. Besag J, Clifford P. Generalized Monte Carlo significance tests. Biometrika. 1989;76(4):633–42.

52. Fosdick BK, Larremore DB, Nishimura J, Ugander J. Configuring Random Graph Models with Fixed Degree Sequences. SIAM Rev. 2018;60(2):315–55.

53. Hagberg AA, Schult DA. Exploring network structure, dynamics, and function using NetworkX. In: Proceedings of the 7th Python in Science Conference (SciPy2008). 2008. p. 11–5.

54. Federici E, Rossi R, Fidati L, Paracucchi R, Scargetta S, Montalbani E, et al. Characterization of the Skin Microbiota in Italian Stream Frogs (*Rana italica*) infected and uninfected by a cutaneous parasitic disease. Microbes Environ. 2015;30(3):262–9.

55. McKenzie VJ, Bowers RM, Fierer N, Knight R, Lauber CL. Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. ISME J. 2012;6(3):588–96.

56. Abarca JG, Vargas G, Zuniga I, Whitfield SM, Woodhams DC, Kerby J, et al. Assessment of bacterial communities associated with the skin of Costa Rican amphibians at La Selva Biological Station. Front Microbiol. 2018;9:1–12.

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412300 — This a preprint and has not been peer reviewed. Data may be preliminary.

57. Belden LK, Hughey MC, Rebollar EA, Umile TP, Loftus SC, Burzynski EA, et al. Panamanian frog species host unique skin bacterial communities. Front Microbiol. 2015;6:1–21.

58. Bletz MC, Archer H, Harris RN, McKenzie VJ, Rabemananjara FCE, Rakotoarison A, et al. Host ecology rather than host phylogeny drives amphibian skin microbial community structure in the biodiversity hotspot of Madagascar. Front Microbiol. 2017;8:1–14.

59. Varela BJ, Lesbarrères D, Ibáñez R, Green DM. Environmental and host effects on skin bacterial community composition in Panamanian frogs. Front Microbiol. 2018;9:1–13.

60. Becker MH, Walke JB, Murrill L, Woodhams DC, Reinert LK, Rollins-Smith LA, et al. Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis* . Mol Ecol. 2015;24(7):1628–41.

61. Brucker RM, Baylor CM, Walters RL, Lauer A, Harris RN, Minbiole KPC. The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander*Plethodon cinereus* . J Chem Ecol. 2008;34(1):39–43.

62. Amend AS, Seifert KA, Bruns TD. Quantifying microbial communities with 454 pyrosequencing: Does read abundance count? Mol Ecol. 2010;19(24):5555–65.

63. Hernández-Gómez O, Briggler JT, Williams RN. Influence of immunogenetics, sex and body condition on the cutaneous microbial communities of two giant salamanders. Mol Ecol. 2018;27(8):1915–29.

64. Deveau A, Bonito G, Uehling J, Paoletti M, Becker M, Bindschedler S, et al. Bacterial-fungal interactions: Ecology, mechanisms and challenges. FEMS Microbiol Rev. 2018;42(3):335–52.

65. Frey-Klett P, Garbaye J. Mycorrhiza helper bacteria: A promising model for the genomic analysis of fungal-bacterial interactions. New Phytol. 2005;168(1):4–8.

66. Walke JB, Belden LK. Harnessing the microbiome to prevent fungal infections: Lessons from amphibians. PLoS Pathog. 2016;12(9):6–11.

67. Rowley JJL, Gleason FH, Andreou D, Marshall WL, Lilje O, Gozlan R. Impacts of mesomycetozoean parasites on amphibian and freshwater fish populations. Vol. 27, Fungal Biology Reviews. 2013. p. 100–11.

68. Badali H, Bonifaz A, Barrn-Tapia T, Vzquez-Gonzlez D, Estrada-Aguilar L, Cavalcante Oliveira NM, et al. Rhinocladiella aquaspersa, proven agent of verrucous skin infection and a novel type of chromoblastomycosis. Med Mycol. 2010;48(5):696–703.

69. Seyedmousavi S, Guillot J, Tolooe A, Verweij PE. Neglected fungal zoonoses: hidden threats to man and animals. Clin Microbiol Infect. 2015;21:416–25.

70. Gram L, Melchiorsen J, Spanggaard B, Huber I, Al GET, Icrobiol APPLENM. AH2 , a possible probiotic treatment of fish. 1999;65(3):969–73.

71. Ghadban GS. Probiotics in broiler production - A review. Arch fur Geflugelkd. 2002;66(2):49–58.

72. Cheng TL, Mayberry H, McGuire LP, Hoyt JR, Langwig KE, Nguyen H, et al. Efficacy of a probiotic bacterium to treat bats affected by the disease white-nose syndrome. J Appl Ecol. 2017;54(3):701–8.

73. Belasen AM, Riolo MA, Lyra ML, Toledo LF, James TY. 2020. *Thoropa taophora* genetic

and microbiome data. Dryad [identifier to be added upon article acceptance].

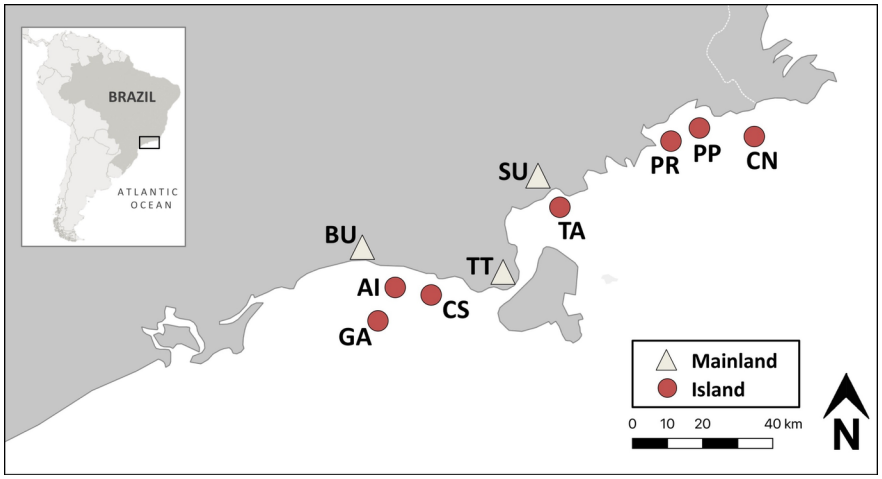
Data Accessibility

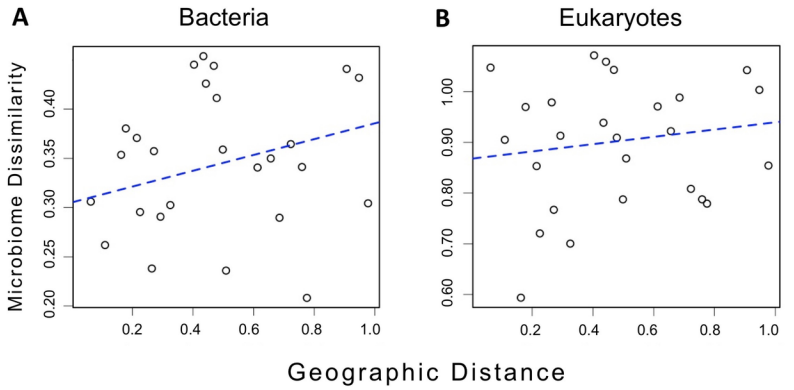
Genetic data including MHC sequences, 18S sequences, and 16S sequences have been accessioned in GenBank (accession numbers to be added upon publication).

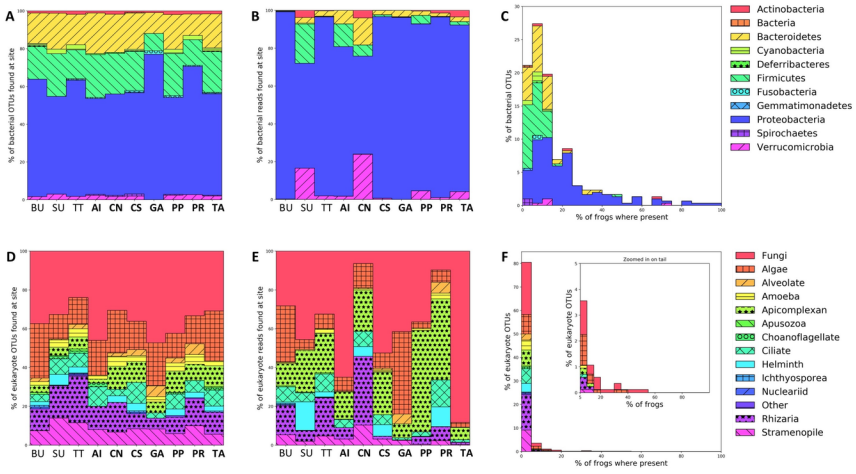
Author Contributions

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412300 — This a preprint and has not been peer reviewed. Data may be preliminary.

AMB and TYJ conceived of the study, AMB, LFT, and TYJ did the fieldwork, AMB and MLL did the labwork, AMB and MAR analyzed the data, AMB wrote the manuscript, all authors assisted in manuscript preparation and editing.

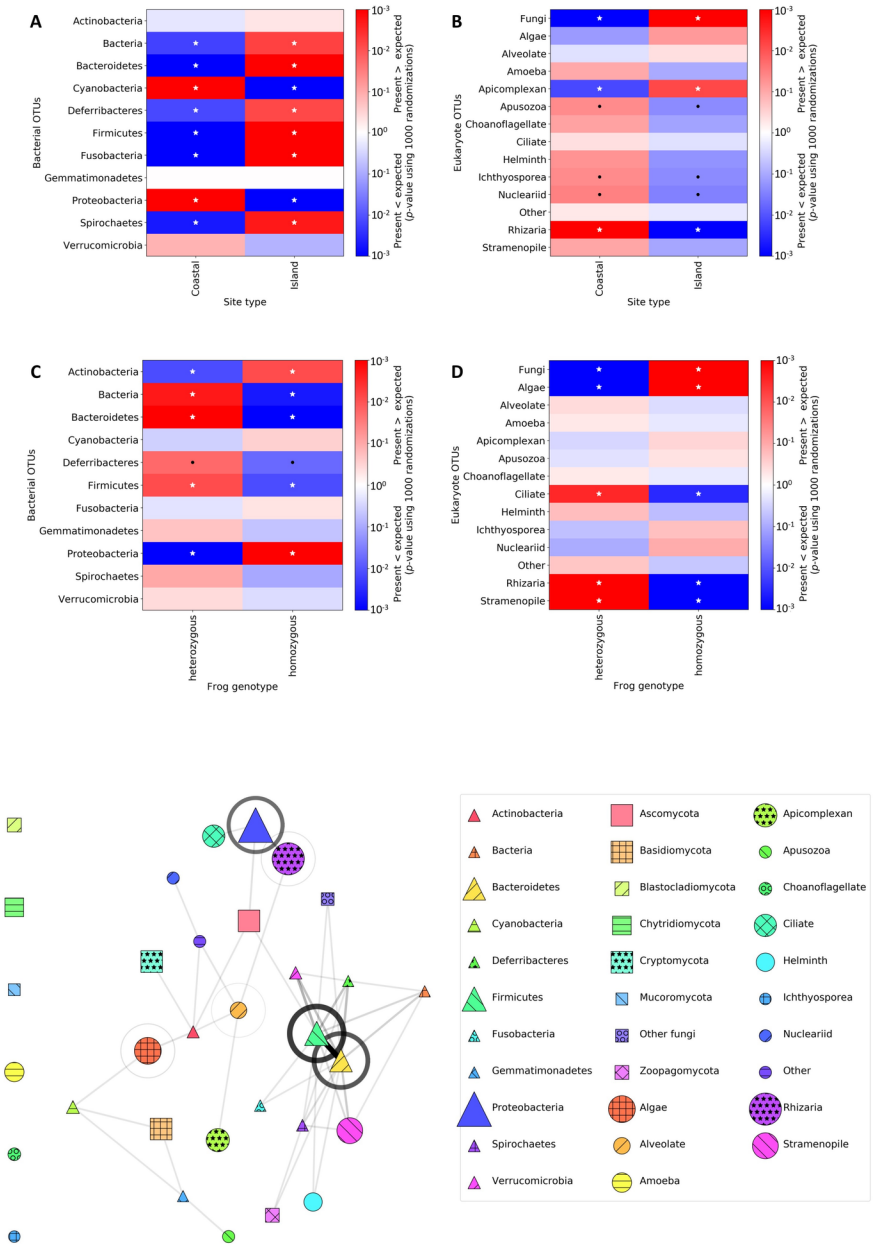


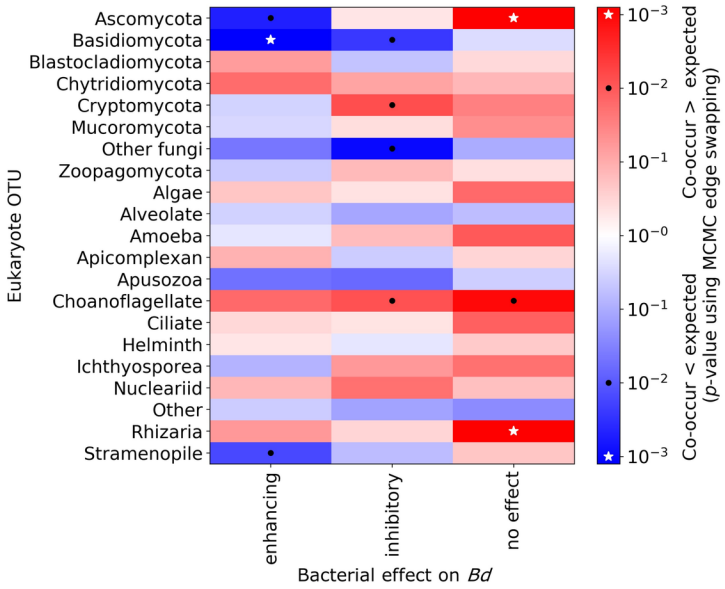




Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412800 — This a preprint and has not been peer reviewed. Data may be preliminary.

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412600 — This a preprint and has not been peer reviewed. Data may be preliminary.





Hosted file

Table 1 9jan20.docx available at https://authorea.com/users/318073/articles/448044-geography-host-genetics-and-cross-domain-microbial-networks-structure-the-skin-microbiome-of-fragmented-brazilian-atlantic-forest-frog-populations

Posted on Authorea 6 May 2020 — CC BY 4.0 — https://doi.org/10.22541/au.158880278.82412300 — This a preprint and has not been peer reviewed. Data may be preliminary.