

ARTICLE

Microbial Ecology

Differential skin microbiomes and disease outcomes in sympatric hosts despite similar infection burdens

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Abstract

Amphibians are declining worldwide, in large part due to chytridiomycosis: a disease of amphibian skin caused by the fungal pathogen, *Batrachochytrium dendrobatidis* (Bd). Resistance and tolerance to Bd infection can exhibit intra- and interspecific variation. This variation may depend, among other factors, on the host skin microbiome. In California's Sierra Nevada, endangered mountain yellow-legged frogs (MYL frogs, *Rana sierrae* and *Rana muscosa*) are highly susceptible to chytridiomycosis, while the co-occurring Pacific chorus frog (*Pseudacris regilla*) is less susceptible and may function as a Bd reservoir host. To investigate whether distinct Bd dynamics for these sympatric hosts relate to differences in skin microbial communities, we (1) compared Bd infection of MYL frogs and *P. regilla* across 23 Sierra Nevada sites, and (2) characterized the bacterial component of host skin microbiomes (or bacteriomes). Bd infection prevalence was approximately 80% in both frog species, and average infection intensities on *P. regilla* and MYL frogs did not differ. In contrast, bacteriome compositions were markedly distinct. *P. regilla* had a more diverse skin bacteriome than MYL frogs, and only MYL frog bacteriome diversity correlated with Bd infection intensity. Additionally, the *P. regilla* bacteriome contained a higher richness of putative antifungal bacteria than MYL frogs. Differences in the bacteriomes of these sympatric hosts may be due to differences in physiology or life histories. Further work will be important in determining whether the greater diversity and apparent stability of the *P. regilla* microbiome is a cause or consequence of the host's innate Bd tolerance.

KEYWORDS

amphibian, *Batrachochytrium dendrobatidis*, disease, enzootic, microbiome, *Pseudacris regilla*, *Rana sierrae*, Sierra Nevada

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INTRODUCTION

Amphibians are facing declines at alarming rates due to habitat loss and fragmentation, overexploitation, climate change, and disease (Luedtke et al., 2023; Scheele et al., 2019; Wake & Vredenburg, 2008). Global emergence of the fungal pathogen, *Batrachochytrium dendrobatidis* (Bd), and its resulting disease, chytridiomycosis, has led to declines and extinctions of hundreds of amphibian populations and species (Scheele et al., 2019; Skerratt et al., 2007; Voyles et al., 2009). Bd infects the keratinized skin on amphibians via aquatic zoospores (Berger et al., 2005; Longcore et al., 1999) and, despite its high virulence and transmission rates, Bd does not affect all amphibian hosts equally. While many amphibian species and populations have declined due to Bd, others have remained abundant and widespread, resisting or tolerating infection (Fisher et al., 2009; Voyles et al., 2011). Other impacted species are adapting and recovering, both naturally and as a result of management actions (Knapp et al., 2024).

Understanding and predicting species- or population-level differences in the outcomes of Bd infection requires knowledge of the potential mechanisms controlling Bd susceptibility. Amphibians' defenses may include the bacterial component of their skin microbiome (bacteriome, hereafter), and the potential of those bacteria to limit the growth of pathogenic fungi. Symbiotic skin bacteria may facilitate survival against Bd by producing antifungal compounds (Becker et al., 2015; Harris et al., 2009) or by competing for microbial niche space across the amphibian skin (Rebollar et al., 2020). Bacterial richness and diversity can correspond antagonistically with Bd infection intensity, both in controlled laboratory settings and in wild amphibian hosts (Bates et al., 2018; Becker et al., 2015; Greenspan et al., 2022; Piovita-Scott et al., 2017; Rebollar et al., 2016). Nonetheless, antifungal benefits of bacteria are not equal among amphibian host species (Rebollar et al., 2020).

In California's Sierra Nevada mountains, several anuran species are known to be infected by Bd. Endangered mountain yellow-legged frogs (MYL frogs), *Rana sierrae* and *Rana muscosa*, are closely related sister species (once considered the same species), that have been extirpated from approximately 93% of their range: primarily by the emergence of Bd and the introduction of non-native predatory fish (Bradford et al., 1994; Knapp et al., 2007; Knapp & Matthews, 2000; Vredenburg et al., 2007, 2010). *R. sierrae* and *R. muscosa*, which will be collectively referred to as "MYL frogs," have known similarities in ecology and disease dynamics (Vredenburg et al., 2007, 2010; Wilber et al., 2022); both are highly susceptible to Bd infection (Rachowicz et al., 2006) and

chytridiomycosis drives ongoing declines (Knapp et al., 2022; Vredenburg et al., 2010). However, even among MYL frog populations, divergent outcomes of pathogen and disease emergence occur (Wilber et al., 2022). Some populations experience mass mortality events following the emergence of disease (i.e., epizootics), while other populations persist at near-historic densities with relatively low levels of infection (i.e., in an enzootic state) (Briggs et al., 2010; Vredenburg et al., 2019). Due to their dramatic population declines, studies on disease drivers and host resilience in Sierra Nevada amphibians have focused on MYL frogs, while Bd in other Sierra Nevada amphibians has received less research attention. Widescale measurement of Bd in anuran species that co-occupy sites alongside MYL frogs has not occurred. These data are important to seek, as co-occurring Bd host species potentially influence the relationship between Bd and MYL frogs.

The Pacific chorus frog, *Pseudacris regilla*, is one of the most abundant and widespread amphibians in western North America (Figure 1) (Brattstrom & Warren, 1955; CDFW Open Data Portal, 2016). *P. regilla* individuals are capable of carrying Bd infection intensities—or loads (i.e., zoospore equivalents)—much higher than Bd loads that are lethal to MYL frogs (Reeder et al., 2012; Vredenburg et al., 2010). Their ability to maintain high infection levels across a broad distribution that overlaps with most western amphibians, many of which are susceptible to Bd, has led to the suggestion that *P. regilla* acts as a Bd reservoir host (Gervasi et al., 2013; Padgett-Flohr & Hopkins II, 2009; Reeder et al., 2012). In this context, a Bd reservoir is defined as a tolerant species that can be infected with Bd, but does not show symptoms of chytridiomycosis (Reeder et al., 2012). In the Sierra Nevada, *P. regilla* commonly occur in habitats where MYL frogs were extirpated by Bd epizootics, and habitats where MYL frogs remain extant (Bradford et al., 1994; Briggs et al., 2010; Knapp, 2005; Knapp et al., 2024; Reeder et al., 2012). The interactions between these sympatric amphibian species, in terms of Bd infection and transmission, are not yet understood.

Early studies of MYL frogs suggested that pre-existing symbiotic bacteria may contribute to innate immune responses that protect against Bd infection (Woodhams et al., 2007). However, in both natural settings and the laboratory, there is strong evidence that Bd infection disrupts the skin bacteriome of *R. sierrae*, and that the component skin bacterial taxa are differentially impacted by increasing Bd infection (Jani et al., 2021; Jani & Briggs, 2014). Additionally, *R. sierrae* populations that exhibit different disease dynamics (enzootic vs. epizootic) are associated with distinct skin bacterial communities, and these differences in bacterial composition (i.e., the

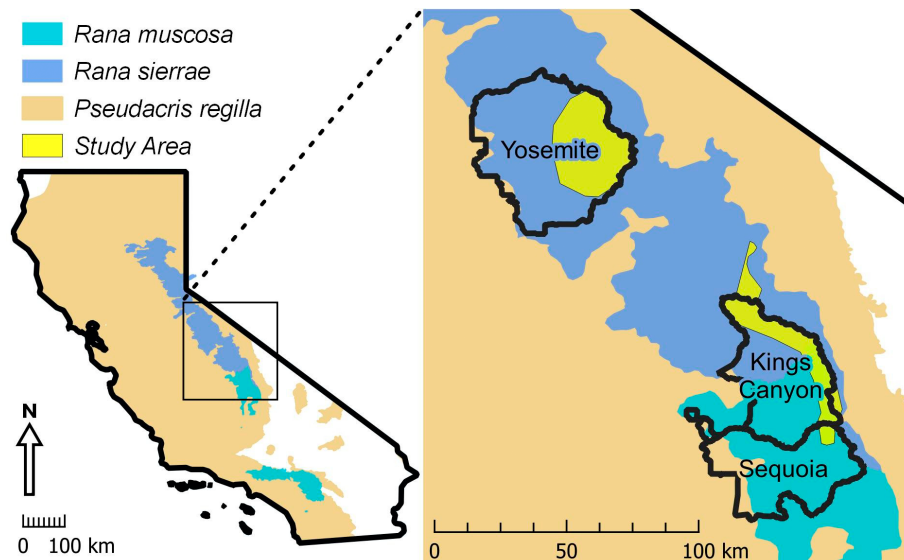


FIGURE 1 Map of study species' distributions and study area in California, USA. Enlarged area displays the region in which study lakes are located and, for orientation, displays the national parks where most of the study occurred. *Pseudacris regilla* map is adapted from California Department of Fish and Wildlife (CDFW) (2016).

number and identity of bacterial members) do correlate with Bd load (Jani et al., 2017).

Because *P. regilla* can carry very high loads of Bd and co-occur with declining amphibians, this species has the potential to impact susceptible species. On one hand, *P. regilla* may pose a threat to other amphibians due to its potential role as a Bd reservoir. On the other hand, if the *P. regilla* skin bacteriome plays a role in its tolerance to Bd infection, then transmission of bacterial members among sympatric hosts could potentially buffer susceptible species from disease. Yet the role of the *P. regilla* skin bacteriome in disease resistance is not known, nor do we understand the connections between the microbiota of *P. regilla* and sympatric amphibians. Very few studies have investigated *P. regilla* skin microbial communities (Kueneman et al., 2014), and even fewer have investigated the relationships between *P. regilla* skin microbes and Bd infection (Basanta et al., 2022).

Our limited knowledge of the *P. regilla* skin bacteriome and Bd tolerance highlights the need to ask whether this proposed Bd reservoir could be impacting vulnerable co-occurring species. In this study, we leveraged natural variation in species co-occurrence to investigate *P. regilla* Bd infections and skin bacterial communities at a landscape scale, and to examine the potential effects of *P. regilla* on MYL frog populations. We investigated *P. regilla* Bd infection prevalence and intensity, and characterized the skin bacteriome of *P. regilla* in the southern extent of *R. sierrae*'s range. We then compared the skin bacterial communities of MYL frogs and *P. regilla* across the same range of habitat, and

examined whether bacterial communities correlated with Bd infection in the Bd susceptible and purported Bd reservoir hosts. We predicted that *P. regilla* Bd loads would be higher than loads observed in MYL frogs, and that skin bacterial communities would be distinct between these amphibian hosts. If the skin bacteriome contributes to *P. regilla*'s Bd tolerance, we expected that *P. regilla* skin bacteriomes would contain greater bacterial richness, including a higher richness of putative antifungal bacteria, compared to MYL frogs. Given their historic co-occurrence with Bd susceptible *R. sierrae* and *R. muscosa*, and their occupancy in sites where MYL frog recovery actions are ongoing or planned, this study is important for understanding Bd infections in *P. regilla* throughout the Sierra Nevada, and further understanding this species' potential role as a Bd reservoir.

METHODS

To investigate relationships between Bd infection levels and skin bacteriomes in co-occurring Sierra Nevada amphibians, our objectives were to (1) compare Bd infection prevalence and intensity of *P. regilla* and MYL frogs at a subset of Sierra Nevada sites, (2) profile *P. regilla* and MYL frog skin bacteriomes at these same sites, (3) explore correlations between skin bacteriome compositions and Bd infection loads for both hosts, and (4) determine the richness of putative Bd-inhibitory bacterial taxa within the skin bacteriome communities of *P. regilla* and MYL frogs.

Field sampling: Amphibian surveys and swab collection

One goal of this study was to sample sites of single-amphibian species occupancy (i.e., MYL frogs or *P. regilla*), as well as sites where both amphibians co-occurred (i.e., MYL frogs and *P. regilla*). From July to September of 2023, we sampled 23 sites throughout the central and southern Sierra Nevada with known occupancy of MYL frogs and/or *P. regilla*. Of the sampled sites, 11 were in Yosemite National Park, three were in Inyo National Forest, one was in Sierra National Forest, and eight were in Sequoia-Kings Canyon National Park (Figure 1). Study sites (waterbodies) ranged in elevation from 2957 to 3495 m, maximum depths of 0.5 to 17.8 m, surface areas of 43 to 40,331 m², and were surrounded by subalpine or alpine vegetation. Each site was visited once during the summer season.

Sampled sites where MYL frogs occurred were populations characterized by enzootic Bd dynamics (persisting populations with low Bd loads: Briggs et al., 2010), with the exception of four sites that were designated as Bd-free. These site designations were characterized based on multi-decade population survey and disease data collection (Smith & Knapp, unpublished; Wilber et al., 2022). We did not know the Bd status of single-occupancy *P. regilla* sites prior to sampling. Of the four Bd-free sites we sampled, two were co-occupied by *R. muscosa* and *P. regilla*. For our study, *R. sierrae* and *R. muscosa* data are combined based on known similarities in ecology and disease dynamics (Vredenburg et al., 2007, 2010; Wilber et al., 2022), and analyses refer to them as “MYL frogs” unless otherwise stated. As the majority of our analyses focused only on infected frogs, and all *R. muscosa* individuals sampled were Bd-negative, comparisons between frog species are comparisons between *R. sierrae* and *P. regilla*, and are stated as such.

Fully post-metamorphic frogs (tail fully resorbed, Gosner stage 46; snout-vent length (SVL) *P. regilla* >20 mm; MYL frog >32 mm) were captured either by hand or dipnet, and each individual was handled with a new pair of nitrile gloves. To avoid re-sampling the same individuals, MYL frog adults (>40 mm) were scanned and identified by passive integrative transponder (PIT) tags (8 mm, Biomark) (at long-term monitoring sites). MYL frog subadults (32–39 mm) were untagged but caught infrequently, and at sites with a large spatial area, where re-sampling was not a concern. *P. regilla* were untagged; therefore, to avoid re-sampling, individual *P. regilla* were each held in separate, clean plastic containers, along with a small amount of lake water, and were not released until sampling was complete. In order to remove transient microbes from frog skin, we rinsed

each individual with 60 mL of filter-sterilized lake water prior to sampling (Culp et al., 2007; Jani & Briggs, 2018; McKenzie et al., 2012). Following rinsing, we collected two samples using sterile synthetic swabs (MW113, Medical Wire and Equipment) to capture skin bacterial communities and frog skin cells containing Bd zoospores. We additionally measured SVLs and masses for each frog. Skin bacteriome samples were collected by swabbing the entire body (dorsal and ventral) of the individual for 30 s (Kueneman et al., 2014; McKenzie et al., 2012). Bacteriome swabs were stored in 50 µL DNA/RNA shield (Zymo Research) to preserve DNA while in the field. Bd samples were collected following a standard swabbing protocol (ventral surface only): strokes were made on the left and right sides of the abdomen five times each, left and right thighs five times each, and left and right rear feet five times each for a total of 30 strokes (Briggs et al., 2010; Hyatt et al., 2007).

Bd swabs were fully air-dried in the field (~45 min), away from the sampling area, before being stored in individual 1.5-mL sterile microcentrifuge tubes (Andwin Scientific), as the remote location of sites prevented rapid transport and freezing of swabs (Hyatt et al., 2007). Skin bacteriome swabs were always collected first; previous work has shown that Bd samples are not impacted by collecting bacteriome samples prior to Bd samples (Jani et al., 2021). Bd and skin bacteriome swab samples were frozen at −20 and −80°C, respectively, upon returning to the laboratory. To capture the planktonic microbial communities that may serve as a source of bacterial colonists for frog skin bacteriomes, we collected two water filtrate samples at each site, with the exception of four sites where we collected a single sample and one site where we collected three samples. Variation in filter sample numbers was due to variation in water chemical composition that affected the amount of water we could filter in the time available (see Appendix S1 for additional methods and results).

Bd quantification and infection analyses

Bd samples were processed at the University of California Sierra Nevada Aquatic Research Laboratory molecular diagnostics laboratory in Mammoth Lakes, CA (doi: 10.21973/N3966F). DNA was extracted from swabs using Prepman Ultra (Applied Biosystems). Bd infection intensity was quantified by performing a standard quantitative polymerase chain reaction (qPCR) assay (Boyle et al., 2004; Hyatt et al., 2007), with samples run in singlicate (Knapp et al., 2023; Kriger et al., 2006; Yap et al., 2016). We chose to maximize biological replicates (number of frogs swabbed per site) rather than technical

replicates (PCRs per swab) because biological replicates provide more information toward understanding differences among sites or species. Bd load (reported as the number of ITS1 gene copies per swab) was calculated by multiplying the raw qPCR output by 80 to account for the dilution that occurs during qPCR reactions (Joseph & Knapp, 2018; Vredenburg et al., 2010). We report Bd loads as ITS1 gene copies; although the number of ITS1 copies varies across Bd strains (Longo et al., 2013), Bd from MYL frogs in prior studies revealed an average of 60 ITS1 gene copies per zoospore (Joseph & Knapp, 2018).

All Bd summary calculations and analyses were conducted using R version 4.3.2 (R Core Team, 2023). Bd prevalence was calculated as the percentage of infected animals from the total number of sampled individuals at each site. We constructed a binomial model and used a likelihood ratio test (LRT) to compare Bd infection prevalence between frog species. We performed a $\log_{10}$ transformation [$\log_{10}(\text{Bd infection intensity} + 1)$] and used ANOVA to compare average Bd infection intensities of infected frogs across sites. We additionally used a Welch's two sample *t* test to compare the average Bd load between infected MYL frogs and *P. regilla* and constructed linear models to compare Bd loads between infected host species at sites where they co-occurred (Appendix S1: Table S1).

16S amplicon sequencing for determining bacterial diversity and community composition

Microbial samples were processed in the Genomics/Transcriptomics Analytics Core facility at San Francisco State University. DNA extraction was performed utilizing the IBI gMax Mini Genomic DNA kit (IBI Scientific). For skin bacteriome swabs, the IBI extraction protocol was modified for use with 50 μL Zymo DNA/RNA shield, by the addition of an initial lysozyme digestion step prior to the proteinase-K digestion step. Extracted DNA was eluted in 100 μL elution buffer (IBI). DNA extracts were kept at 4°C for short-term storage, and -20°C for long-term storage.

The V4 region of the 16s rRNA gene was amplified using barcoded primers (515FB/806RB) and PCR conditions adapted from Caporaso et al. (2018). Our 25 μL PCRs contained 4.5 μL H₂O, 10 μL Platinum Hot Start PCR Master Mix (2 $\times$), 5 μL 1 μM forward primer, 0.5 μL 10 μM reverse primer, and 5 μL sample DNA. Reactions were held at 95°C for 5 min to activate Taq and denature DNA, followed by 35 amplification cycles at 95°C for 30 s, 50°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 5 min. A randomized subset of samples was visualized on a 1% agarose gel to confirm amplification.

PCR products were cleaned using Serapure magnetic beads (Rohland & Reich, 2012) and eluted in 20 μL elution buffer (Qiagen). Clean DNA was quantified using Quant-It Picogreen dsDNA reagent in a 384-well plate on a BioTek Synteny LX Multimode Microplate Reader. For samples that showed very low (or zero) DNA concentration, the previous amplification and cleaning steps were repeated in triplicate, and products pooled.

After cleaning and quantification of all samples, two separate libraries were prepared: one containing bacterial DNA from all individual frog skin bacteriome samples, and one containing all site-level planktonic bacterial DNA. To prepare each library, all samples were normalized by diluting to 2–5 ng/ μL , and amplicons pooled. The total DNA concentration of each library was quantified using qPCR with Illumina primers (KAPA Library Quantification Kit ABI Prism, Roche Sequencing & Life Science) on an ABI StepOne Real Time PCR System. Each library was sequenced in the forward and reverse directions on an Illumina MiSeq using the MiSeq Reagent Kit v3 (600 cycles; PE-300, Illumina).

Bioinformatics to describe microbial diversity

Base calling and demultiplexing were performed by Illumina MiSeq Reporter Software. Bioinformatics analyses were carried out using QIIME2 2024.2 (Bolyen et al., 2019) unless otherwise stated. For each MiSeq run, paired-end sequences were processed into amplicon sequence variants (ASVs) using the *dada2* package (Callahan et al., 2016). Default parameters were used to filter low-quality reads and perform denoising. Forward and reverse reads were trimmed at 250 and 95 bp, respectively, due to a spontaneous and significant drop in quality in the reverse reads. Paired sequences were merged and chimeric sequences were removed using *-p*-min-fold-parent-over-abundance set to “4.” At this point, data from both sequencing runs (frog skin and planktonic bacteria) were merged together to allow for consistency in downstream analyses. Taxonomy was assigned using a naive Bayesian classifier (Wang et al., 2007) and the SILVA v138 database (Bokulich et al., 2018; Quast et al., 2013). Mitochondrial and chloroplast ASVs were discarded, as were any sequences identified as eukaryote, archaea, or unidentified at the phylum level. The *microDecon* package in R, which calculates proportions of contaminant ASVs from control samples, was used to identify and remove potential contaminant sequences from our sample data (McKnight et al., 2019). Sequences were aligned using MAFFT, and a phylogenetic tree was created using FastTree. Stacked barplots

were produced to visualize the identities and relative abundances of bacterial taxa.

After quality control, filtering, and denoising, our dataset contained 293 samples: *P. regilla* $n = 112$, MYL frogs $n = 140$, planktonic microbes $n = 41$. Our data retained 14,024,114 reads with a total of 26,346 ASVs. We subsampled to 8176 sequences per sample, based on the point at which taxon accumulation (rarefaction) curves reached an asymptote. Samples with fewer than 8176 sequences were dropped from the analysis to avoid spurious results that can arise due to differences in the number of sequences (“sampling effort”) among samples. The final subsampled ASV table included 20,211 ASVs. We detected 15,769 unique ASVs across 252 amphibian swabs, and 8013 unique ASVs in 41 planktonic microbial samples. *P. regilla* had an average of 271 ASVs/sample, and MYL frogs had an average of 163 ASVs/sample.

Subsampled data was used for all diversity analyses. We quantified alpha diversity by calculating Shannon diversity, Faith’s phylogenetic diversity, Pielou’s evenness, and richness (observed number of ASVs) using the q2-diversity plugin. Alpha diversity metrics were compared using a Kruskal-Wallis test. Beta diversity was quantified using weighted and unweighted UniFrac distances. To test for differentially abundant bacterial taxa present on amphibian host species, analysis of compositions of microbiomes with bias correction (ANCOM-BC) was used on filtered, but non-subsampled, data (Lin & Peddada, 2020). To identify putative Bd-inhibitory ASVs, we used q2-vsearch (Rognes et al., 2016) with a closed reference feature clustering strategy to compare our sequences against a database of known 16S rRNA sequences from anti-Bd bacteria (Woodhams et al., 2015). Clustering occurred at 99% similarity. Unless otherwise stated, all remaining downstream analyses were conducted in R.

Statistical analyses of bacterial diversity

We explored differences in alpha diversity indices (Shannon diversity, Faith’s phylogenetic diversity (PD), evenness, and richness) relative to (1) species (*P. regilla* and MYL frogs), (2) Bd infection status within each host species, and (3) Bd load within each host species. The Faith’s PD, evenness, and richness diversity data were not normally distributed, so we used nonparametric methods to explore these relationships: a Wilcoxon rank sum test for categorical comparisons and Spearman’s rank correlation to test non-causal relationships between alpha diversity indices and Bd infection load ($\log_{10}$ ITS1 gene copies) of infected frogs only.

The Shannon diversity index incorporates richness and evenness information, and was normally distributed

for our data. To understand how host and environmental factors may contribute to bacterial diversity, we constructed linear mixed-effects models with Shannon diversity as the response variable and Bd load, host species, and elevation as predictors. While other environmental variables such as lake area and lake depth may be important factors that affect bacterial diversity or Bd–bacteriome relationships, we could not measure those variables for every site. Rather, we accounted for any site-level variation by testing models with “site” ($n = 23$) as a random effect. We also included an interaction between Bd load and frog species to enable detection of different responses to Bd load between host species. We compared nested mixed effects models using the *performance* package (Lüdtke et al., 2021) and Akaike information criterion (AIC)-based model selection criteria, with a minimum improvement of two units AIC to justify keeping a variable. We ultimately selected the model with the lowest AIC score as the best-fit model (Zuur et al., 2009) (Appendix S1: Tables S2 and S3).

We investigated differences in bacterial community composition using weighted and unweighted UniFrac distances. The UniFrac metric accounts for differences in phylogenetic relationships; weighted UniFrac accounts for the relative abundance of ASVs in the community, while unweighted UniFrac is based only on the presence/absence of ASVs (Lozupone et al., 2007; Lozupone & Knight, 2005). Principal coordinate analysis (PCoA) was used to visualize differences among samples. We tested for differences of bacterial composition between amphibian species, and between infected and uninfected frogs, using permutational multivariate ANOVA (PERMANOVA) within the q2-diversity beta-group-significance function. To examine the relationship between Bd infection intensity and bacterial communities across amphibian hosts and sites, the *adonis2* function (PERMANOVA) from the *vegan* package (Oksanen et al., 2001) was used with weighted and unweighted UniFrac distance matrices. We tested the following variables: site + species $\times$ log Bd load.

Differential abundance and putative antifungal bacteria analyses

Within QIIME2, ANCOM-BC was used to identify ASVs that were differentially abundant across amphibian host species and across Bd-infected and uninfected frogs. We used default parameters ($\text{prv-cut} = 0.1$, $p\text{-alpha} = 0.05$, $p\text{-adj} = \text{“holm”}$) on a filtered feature table that removed any samples with library sizes of less than 8176 reads (our read depth for subsampling). To identify potentially “core” taxa that may differ between the two frog species, ANCOM-BC was also run after pooling ASVs at the

genus level and filtering out genera with less than 70% prevalence across samples (prv-cut = 0.7).

To identify putative antifungal bacteria in our samples, we matched our sample ASV sequences to the Amphibac database (Woodhams et al., 2015) at 99% sequence identity. The output was a new ASV table with the number of reads for each putative antifungal sequence in each sample. We then calculated the richness of putative antifungal sequences for each sample. A Spearman's rank correlation was used to test whether the richness of putative antifungal bacteria was correlated with Bd load. We also tested whether the richness of putative antifungal bacteria was different between *P. regilla* and MYL frogs. We used ANCOM-BC in QIIME2 with the antifungal ASV table to determine the differential abundance of putative antifungal ASVs among amphibian host species, at the $p = 0.001$ significance level. A differential abundance bar plot was created after ASVs were pooled at the genus level (or family, if genus was unidentified). We note that these analyses of putatively antifungal bacteria are hypothesis-generating, not hypothesis-testing, because the functional capacity of bacterial strains cannot be determined based solely on a match in the 16S gene sequence.

RESULTS

The objectives of our study were to (1) compare Bd infection prevalence and intensity of *P. regilla* and MYL frogs at a subset of Sierra Nevada sites, (2) profile *P. regilla* and MYL frog skin bacteriomes at these same sites, (3) explore correlations between skin bacteriome compositions and Bd infection loads for both hosts, and (4) determine the richness of putative Bd-inhibitory bacterial taxa within the skin bacteriome communities of *P. regilla* and MYL frogs. Prior to the removal of samples due to quality control steps of our microbial analyses, we collected Bd and skin bacteriome swab samples from *P. regilla* $n = 113$ (SVL 20–48 mm), and MYL frogs $n = 142$ (SVL 32–79 mm) across 23 sites in the Sierra Nevada. Bd was detected at 19 of the 23 sampled sites.

Bd infection prevalence and infection intensity are high in both *P. regilla* and *R. sierrae*

We calculated Bd infection prevalence in both frog genera (*P. regilla* 72.6%, 95% binomial CI 63.4%–80.5%; MYL frogs 57%, 95% binomial CI 48.5%–65.3%). To compare Bd prevalence between frog species, we used a likelihood ratio test to compare a binomial model, with species as the predictor, against the null model. Across our sampled

site types (single-occupancy vs. co-occurring; Table 1), Bd infection prevalence was higher in *P. regilla* than in MYL frogs ($p = 0.0098$). Although Bd is ubiquitous across the Sierra Nevada landscape, few known MYL frog sites remained Bd-free at the time of our study. We chose to sample here to compare skin bacterial communities in uninfected frogs. However, when excluding the four Bd-free sites from Bd prevalence analyses (i.e., only examining Bd prevalence across sites where Bd was detected), Bd prevalence between both species was similar: 80.4% and 80.2% respectively for *P. regilla* ($n = 102$, binomial CI 71.4%–87.6%) and *R. sierrae* ($n = 101$, binomial CI 71.1%–87.5%). Bd was not detected in any of the sites that were assigned Bd-free status a priori; that is, no new epizootics were detected (qPCR of swabs would show high loads of Bd during an outbreak).

Of the infected frogs in both species, Bd infection loads ranged from 115 to 2,021,118 ITS1 gene copies per swab sample and were not different between the two species (Welch's t test, $t = 1.547$, $p = 0.124$) (Appendix S1: Table S1). At sites of co-occurrence, the range of Bd infection intensity for infected *P. regilla* was 874 to 1,093,384 ITS1 gene copies, while the range for infected *R. sierrae* was 391 to 341,965 ITS1 gene copies. We constructed linear models to compare ($\log_{10}$ transformed) Bd loads between infected host species at sites where they co-occurred. The best fitting model had the formula: Bd load $\sim$ species. Based on our model, mean Bd loads of *P. regilla* slightly exceeded *R. sierrae* (*P. regilla* = 4.549 $\log_{10}$ ITS gene copies, *R. sierrae* = 3.634 $\log_{10}$ ITS gene copies, variance = 1.0004, $F = 6.253$ on 1 and 28 df, $p = 0.0185$). We acknowledge that the species coefficients in these pooled analyses partly reflect between-site and between-site occupancy type (single vs. co-occurring) differences. One co-occurring site (Appendix S1: Table S1) was occupied by *R. muscosa* and *P. regilla* and was Bd-free at the time of the study. At this site, Bd was not detected in any swabs from either frog species.

TABLE 1 Summary table of *Batrachochytrium dendrobatidis* (Bd) prevalence and infection intensity across site types (species group vs. co-occurring occupancy).

Site occupancy	No. sites sampled	Bd prevalence (%)	Mean Bd load (ITS1 copies)	Median Bd load (ITS1 copies)
<i>Pseudacris regilla</i>	10	80	76,835.63	4898.2
MYL frog	10	56.5	51,958.61	568.5
Co-occurring	3	54.5	62,168.48	324.2

Abbreviation: MYL, mountain yellow-legged.

Skin bacteriome of *P. regilla* is more diverse than *R. sierrae*, and does not correlate with Bd load

The two host species harbored distinct bacterial communities and showed differences in the relationship between bacteriomes and Bd infection. Overall, *P. regilla* skin bacterial diversity was not correlated with Bd infection and their bacteriomes were more diverse than those of MYL frogs. Alpha diversity was higher in *P. regilla* than MYL frogs for all calculated diversity metrics (richness, Faith's PD, and Pielou's evenness; Wilcoxon rank sum, $p < 0.001$, Appendix S1: Figure S1). Skin bacterial diversity showed no association with Bd load in *P. regilla* for any of the calculated alpha diversity indices (Spearman's rank, all $p > 0.5$), nor did it vary with respect to infection status (infected or uninfected; Wilcoxon rank sum, all $p > 0.05$).

In contrast to *P. regilla*, MYL frog bacterial diversity varied with Bd infection and load. Alpha diversity had a slight but significant negative correlation with Bd load for all diversity metrics (richness, Faith's PD, and Pielou's evenness; Spearman's rank, $p < 0.001$). Richness and evenness were also higher in uninfected versus infected MYL frogs (Wilcoxon rank sum, $p = 0.018$, $p = 0.005$, respectively), while there was no difference in Faith's PD ($p = 0.153$).

Host species and Bd load showed an interactive effect on its relationship to bacterial Shannon diversity. Specifically, Shannon diversity negatively correlated with Bd load in MYL frogs, but not in *P. regilla* (Figure 2; Appendix S1: Tables S2 and S3). While species differences in the relationship between bacterial diversity and Bd load were not formally tested for other alpha diversity metrics (richness, Faith's PD, and Pielou's evenness), the species-specific correlations for these metrics are consistent with a differential relationship.

Skin bacteriome compositions differ between species and sites

Bacteriome composition differed between *P. regilla* and MYL frogs (Figures 3 and 4; weighted UniFrac PERMANOVA pseudo- $F = 75.91$ on 1 and 250 df, $p = 0.001$; unweighted UniFrac PERMANOVA pseudo- $F = 12.45$ on 1 and 250 df, $p = 0.001$). Amphibian skin bacterial communities also differed by site (weighted UniFrac PERMANOVA pseudo- $F = 12.78$ on 22 and 229 df, $p = 0.001$; unweighted UniFrac PERMANOVA pseudo- $F = 3.06$ on 22 and 229 df, $p = 0.001$).

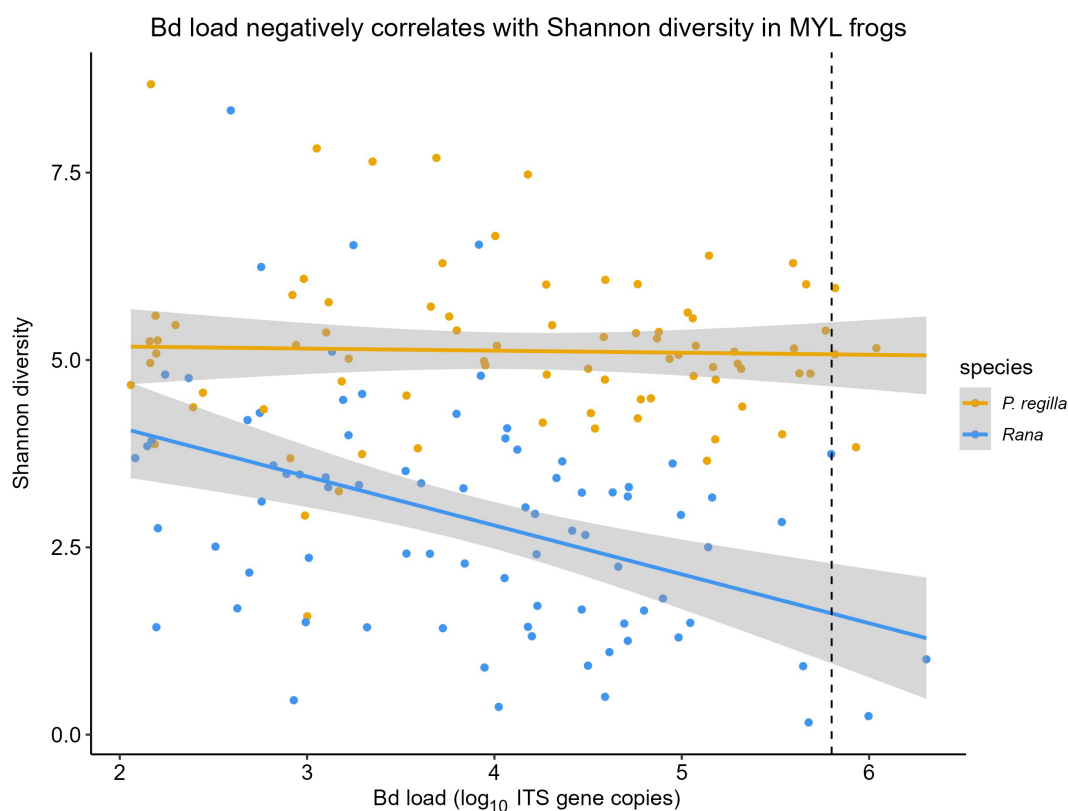


FIGURE 2 As *Batrachochytrium dendrobatidis* (Bd) infection loads increase, skin bacteriome diversity (measured by Shannon diversity) decreases for infected mountain yellow-legged (MYL) frogs (*Rana*), but not for infected *Pseudacris regilla*. Vertical dashed line represents the Bd mortality threshold for *Rana sierrae* (Vredenburg et al., 2010).

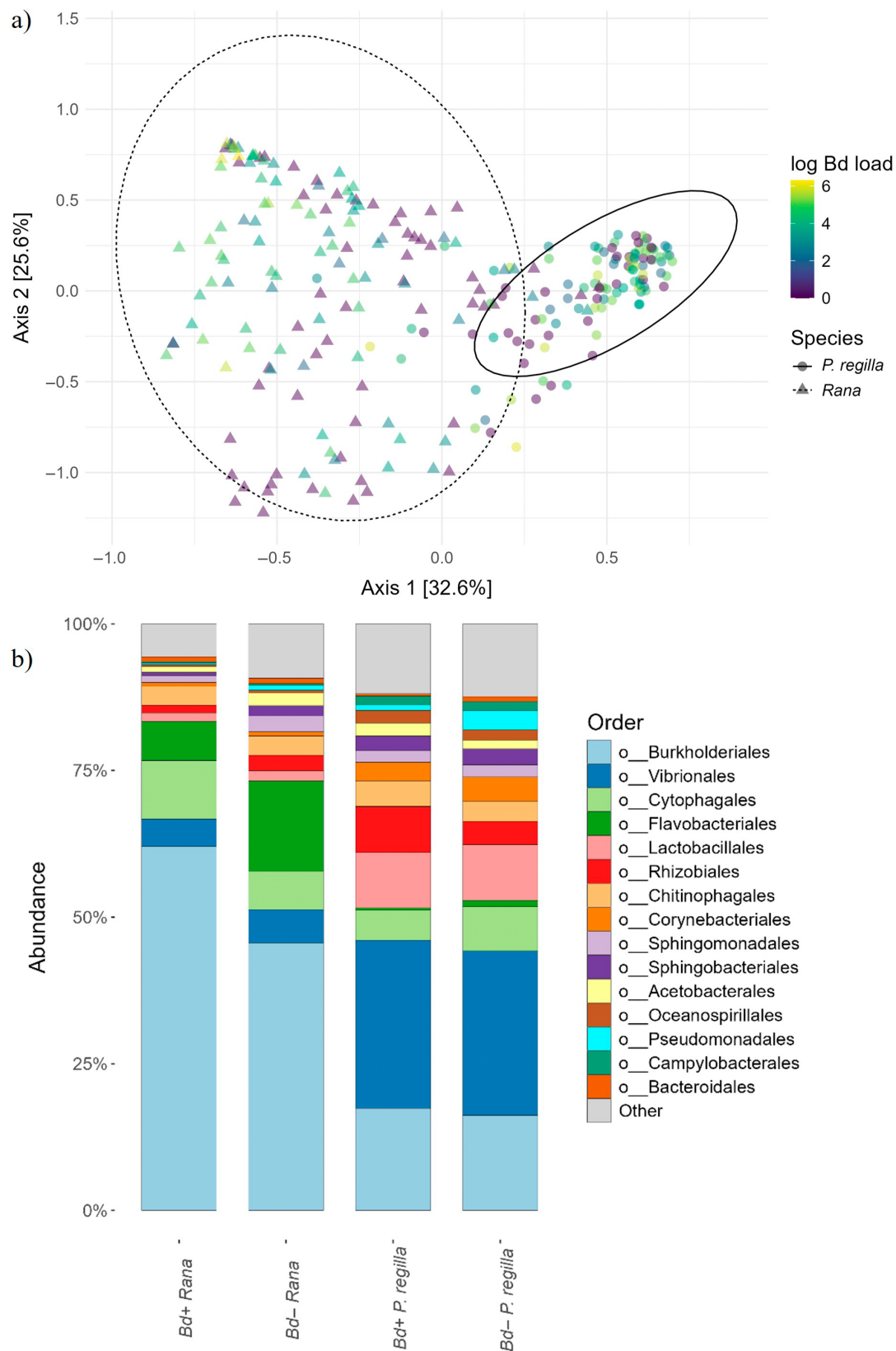


FIGURE 3 (a) Principal coordinates analysis (PCoA) ordination plot with 95% ellipses on weighted UniFrac distance matrix comparing skin bacteriome compositions of mountain yellow-legged (MYL) frogs (*Rana*) and *Pseudacris regilla*. Bacteriome compositions between species are distinct and are not influenced by *Batrachochytrium dendrobatidis* (Bd) infection intensity. For an additional PCoA plot that visualizes Bd-free *Rana muscosa*, see Appendix S1: Figure S3. (b) Relative abundance barplot shows the top 15 bacterial orders present in the skin bacteriomes of infected and uninfected mountain yellow-legged (MYL) frogs (*Rana*) and *P. regilla*. Bacteriome compositions are distinct between frog species and differ based on infection status for MYL frogs.

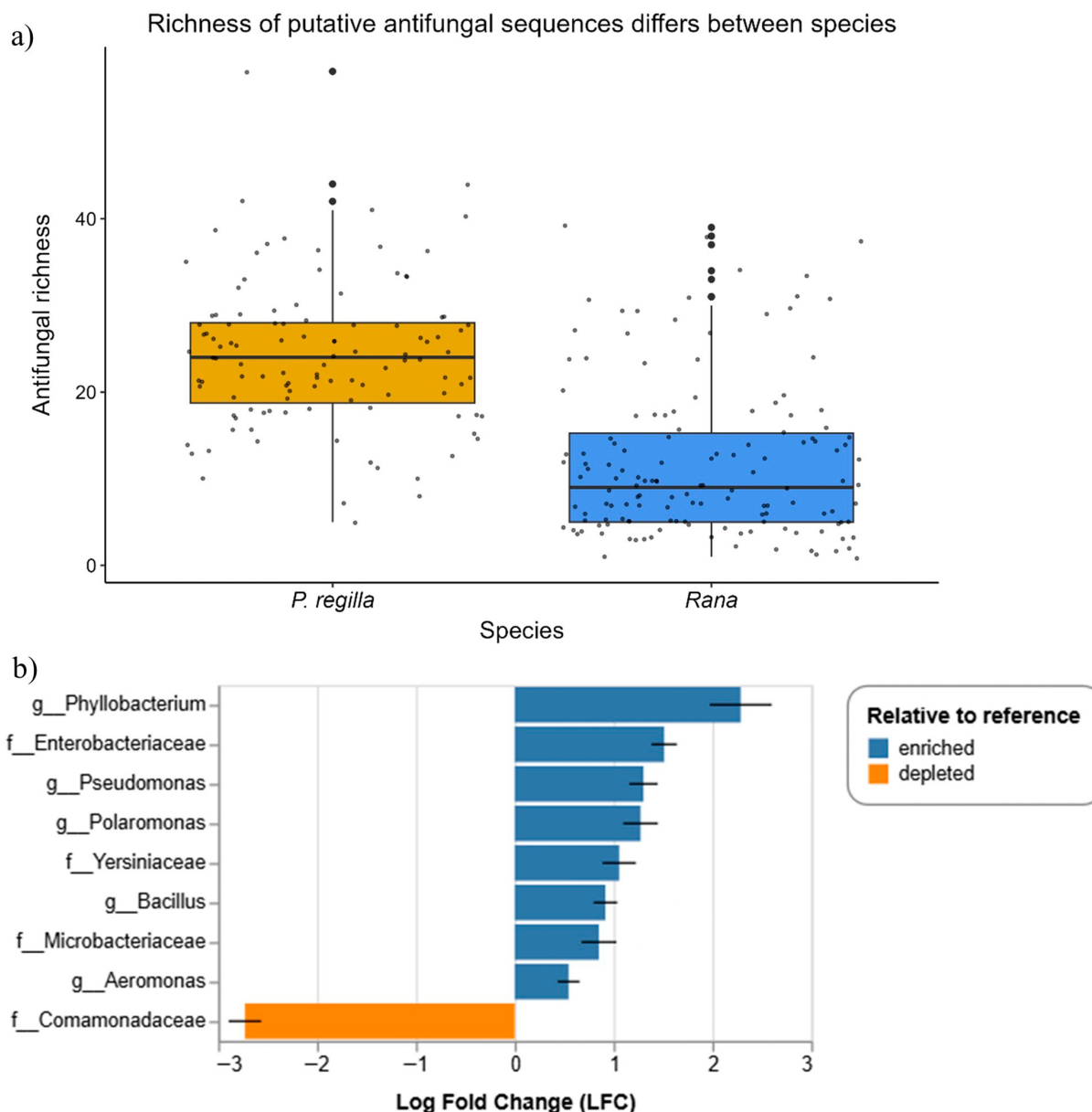


FIGURE 4 (a) Richness (number of amplicon sequence variants [ASVs]) of putative antifungal bacteria detected on *Pseudacris regilla* and mountain yellow-legged (MYL) (*Rana*) frogs. Boxplot midlines show median richness; box limits show interquartile range (middle 50% of values); whiskers show upper and lower 25% of values. Points overlaid on boxplots show values for individual frogs. Outliers are indicated by bold points. (b) Analysis of compositions of microbiomes with bias correction (ANCOM-BC) differential abundance barplot shows the putative antifungal taxa enriched or depleted in *P. regilla* when compared to MYL frogs (*Rana* sp., reference, significance $p = 0.001$). ASVs have been pooled at the genus level. Any taxa unclassified at the genus level have been labeled at the family level (two unclassified *Comamonadaceae* and two unclassified *Enterobacteriaceae* have been grouped).

When we analyzed each species separately, skin bacterial composition of *P. regilla* did not differ based on Bd infection status (weighted UniFrac PERMANOVA pseudo- $F = 1.538$ on 1 and 110 df, $p = 0.148$; unweighted UniFrac PERMANOVA pseudo- $F = 1.074$ on 1 and 110 df, $p = 0.241$). However, MYL frog skin bacterial composition differed between infected and uninfected frogs (weighted UniFrac PERMANOVA pseudo- $F = 7.34$ on 1 and 138 df, $p = 0.001$; unweighted UniFrac PERMANOVA pseudo- $F = 3.27$ on 1 and 138 df, $p = 0.001$), consistent

with our results on bacterial alpha diversity (Figure 2). Amphibian skin bacterial dispersion differed across sites for both weighted and unweighted UniFrac distances (PERMDISP $p < 0.001$) in both species.

We used the *adonis2* (PERMANOVA) function from the *vegan* package to analyze the proportion of variation that could be explained by our variables of interest including Bd load, species, and site. Similar to our univariate analysis for Bd load and alpha diversity, our *adonis2* model did not include explicit covariates for site-related

characteristics, such as lake area or lake depth. Our inputs for the *adonis2* formula followed the model structure: $Y \sim \text{site ID} + \text{species} \times \text{Bd load}$, where our response variable (Y) is the distance matrix of UniFrac distances among each pair of samples. The interaction term between species and infection load was included because our best-fit model of alpha diversity (above) indicated that this interaction may be important. Variation in skin bacteriome communities based on weighted and unweighted UniFrac distances was primarily explained by site and frog species, with little effect from Bd load (Table 2). While site contributed to a large portion of the variation seen in these amphibian bacteriome communities, PERMANOVA (conducted in QIIME2) confirmed that bacterial communities were distinct between frogs and planktonic bacterial communities (weighted and unweighted UniFrac $p = 0.001$) (Appendix S1: Figure S2).

ANCOM-BC (QIIME2) was conducted, at the genus level, on bacterial taxa that occurred in at least 70% of samples to identify potentially “core” taxa for each frog species. This revealed 13 genera (12 named and one unclassified beyond the family level, which was *Comamonadaceae*) that were differentially abundant between *P. regilla* and MYL frogs. Of these taxa, six were enriched in MYL frogs when compared to *P. regilla*, including *Flavobacterium* and the unidentified *Comamonadaceae*. Eight genera were depleted in MYL frogs relative to *P. regilla*, including *Salinivibrio* and *Enterococcus*.

P. regilla has a greater richness of putative antifungal taxa when compared to MYL frogs

Using the Amphibac database (Woodhams et al., 2015), we identified 214 putative antifungal ASVs across 252 amphibian skin swab samples. The *P. regilla* skin

bacteriome had a greater richness of putative antifungal bacteria when compared to MYL frogs (Wilcoxon $p < 0.001$) (Figure 4a). For MYL frogs, the richness of putative antifungal bacteria had a significant, weak negative correlation with Bd load (Spearman's rank $p < 0.001$). However, this relationship was not observed in *P. regilla*; the richness of putative antifungal bacteria in *P. regilla* skin bacteriome communities remained equal across differing infection intensities.

Differential abundance analysis (ANCOM-BC) indicated that of the putative antifungal taxa, *P. regilla* skin bacteriome contained greater abundances of ASVs belonging to the families *Enterobacteriaceae* (two ASVs), *Microbacteriaceae*, and *Yersiniaceae*, and genera *Aeromonas*, *Bacillus*, *Phyllobacterium*, *Polaromonas*, and *Pseudomonas*. MYL frog skin bacteriomes contained a higher abundance of two ASVs unclassified at the genus level, belonging to family *Comamonadaceae* (Figure 4b). These ASVs are unclassified at the genus level because not all bacteria found on the skin of amphibians are represented in the reference databases (e.g., SILVA).

DISCUSSION

Pathogens that infect multiple host species pose challenges to interpreting the impacts of the pathogen on each single host, because infection dynamics in one host species can affect disease risk for others. When one of these hosts is declining, endangered, or subject to management and recovery actions, understanding the multi-host–pathogen relationship takes on greater priority. Reservoir species that maintain infections without developing disease can pose threats, as they can shed infectious pathogens without themselves experiencing severe morbidity or mortality. However, we know of no studies that have co-examined pathogen infection and host-associated skin bacteria in putative reservoir species and co-occurring endangered host species. In this study, we aimed to quantify differences in Bd loads, bacteriomes, and the interaction between Bd and skin bacteria in two sympatric amphibians: *P. regilla* and the *R. sierrae*/*R. muscosa* species complex. Although other amphibian taxa can co-occur, we focused on these hosts because *P. regilla* has been hypothesized to serve as a pathogen reservoir for MYL frogs, and because MYL frogs are threatened and the subjects of heavy investment in recovery.

Our findings are consistent with the hypothesis that *P. regilla* serves as a Bd reservoir for MYL frogs. We quantified Bd prevalence and infection intensity in *P. regilla* and MYL frogs across 23 sites in the Sierra Nevada. Bd prevalence was high in both amphibian

TABLE 2 Permutation test with *adonis* under a reduced model using the unweighted UniFrac distance matrix: Model: unweighted UniFrac $\sim \text{site} + \text{species} \times \text{Batrachochytrium dendrobatidis}$ (Bd) load.

Group	df	SS	R^2	F	Pr(>F)
Site id	18	10.313	0.24036	2.6	0.001
Species	1	0.391	0.00911	1.7	0.005
log(Bd load)	1	0.320	0.00746	1.4	0.042
Species: log(Bd load)	1	0.550	0.01282	2.5	0.001
Residual	140	31.333	0.73024		
Total	161	42.907	1.00000		

Note: This test only examines infected frogs, and the explanatory variables site + species $\times$ Bd load. Site explained a majority of the variation, and there appears to be a small interactive effect between frog species and Bd load.

hosts, and prevalence was similar between amphibian species at sites where Bd is established. In general, Bd infection intensities were moderate and likely sublethal for both host species. Previous laboratory research showed that *P. regilla* can maintain high Bd loads without overt signs of disease (Reeder et al., 2012). This suggested that *P. regilla* could serve as a pathogen reservoir, but the distribution of *P. regilla* Bd loads in the Sierra Nevada was unknown prior to this study.

We found that *P. regilla* and MYL frogs harbored different skin bacteria despite sympatry. Both host and environment are known to influence the amphibian skin bacteriome (Bird et al., 2018; Bletz et al., 2017; Buttner et al., 2021; Kirsch et al., 2024; Kueneman et al., 2014; McKenzie et al., 2012; Walke et al., 2014), and differences in microhabitat may partially explain differences in the skin bacteria of MYL frogs and *P. regilla*. *P. regilla* transition to terrestrial habitats after metamorphosis, while MYL frogs are highly aquatic, even as adults. These differences in host life histories are likely important in the assembly of their bacteriomes. In these distinct habitats (aquatic vs. terrestrial), each species has opportunities to come into contact with different environmental sources of microbes, including soil and vegetation.

In our study, the two frog genera showed different relationships between Bd infection and Shannon diversity. Species-specific analyses of Bd load and additional metrics of bacterial diversity/composition remain consistent with this differential relationship. In MYL frogs, bacterial diversity was higher in uninfected frogs when compared to Bd-infected frogs. In addition, among MYL frogs that were infected with Bd, Bd load was significantly correlated with declines in bacterial richness and changes in bacterial community composition. Bd infection is known to disturb the bacteriome of MYL frogs in both field and laboratory settings (Jani et al., 2017; Jani & Briggs, 2014), and Bd-induced disturbance could explain the relationship between Bd load and bacteriomes of MYL frogs in our study. Interestingly, in *P. regilla*, bacteriome diversity was not predicted by Bd infection status or infection intensity, suggesting that the *P. regilla* bacteriome may be more stable to Bd-induced disturbance. This increased stability could be attributed to characteristics of the bacteriome itself (inherent community stability). Alternatively, it may be explained by the inherent tolerance of the amphibian host species. For example, *P. regilla*'s tolerance to infection could manifest, in part, as reduced pathology of the skin during Bd infection, which could in turn cause less disturbance to the bacteriome. In other words, bacteriome disturbance may be direct (pathogen affects bacterial community) or indirect (pathogen affects host, and host pathology affects bacterial community). While our study cannot

distinguish between these two mechanistic pathways, our results suggest that, among sympatric host species, bacteriome disturbance differs and is linked to host tolerance. This finding highlights the importance of future work to understand the mechanisms underlying disease tolerance and bacteriome stability.

While we found no correlation between bacterial diversity or composition with Bd loads in *P. regilla*, a previous study of *P. regilla* (*hypochondriaca*) populations in Baja, California (Basanta et al., 2022) did detect correlations between bacterial composition (weighted UniFrac distances) and Bd load, with hundreds of ASVs correlating with Bd infection. This difference between Basanta et al. (2022) and our study may be due to dramatic, large-scale environmental differences: Basanta et al. (2022) was conducted in desert, urban, and forest habitats, which are very different environments from the alpine lakes we sampled in the Sierra Nevada. Not only do the frog skin bacteriomes almost certainly differ due to the influence of habitat, but the lineages and characteristics of Bd in these regions may vary and contribute to the observed Bd–bacteriome interactions, highlighting the need for further studies of this widely distributed amphibian species across its entire range.

One mechanism by which the skin bacteriome can bolster host defense against Bd is through the antifungal properties of microbial members. In *R. sierrae*, for example, *Janthinobacterium lividum* can impact Bd growth under certain laboratory conditions (Harris et al., 2009), and has been found on wild *R. sierrae*, although it is quite rare (Jani & Briggs, 2014; Knapp et al., 2022; Lam et al., 2010). Our data show that compared to MYL frogs, *P. regilla* have a higher richness of putative anti-Bd bacteria, and this richness does not change as Bd load increases. Differential abundance analyses also showed that *P. regilla* had much higher abundances of several putative antifungal taxa, relative to MYL frogs. Studies have shown that a greater richness of microbes has been linked to better suppression of Bd (Piovia-Scott et al., 2017). Specifically, a higher richness of putative antifungal bacteria has predicted lower risk of future Bd infection (Chen et al., 2022). However, it is important to avoid over-interpreting analysis of putative antifungal function based on 16S DNA sequence similarity. Putative antifungal bacteria may not all exhibit the same level of Bd inhibition, and relatedness among bacterial taxa does not necessarily indicate a high similarity of anti-Bd function (Antwis et al., 2015; Muletz-Wolz et al., 2017). Given that the prevalence of Bd infection is high across *P. regilla*, and *P. regilla* often have moderate to high Bd infection loads, we hypothesize that antifungal bacteria do not fully prevent or mitigate Bd growth in this species. In order to fully understand the antifungal function

of putative anti-Bd bacteria on *P. regilla*, bacterial culturing and Bd challenge assays would be necessary.

The cause–effect relationship between Bd and the skin bacteriome can be difficult to disentangle: does the skin bacteriome affect Bd infection outcomes, does Bd infection alter the skin bacteriome, or both? For MYL frogs, field surveys and experiments indicate that Bd-induced disturbance is important, yet studies in other amphibian species indicate that the bacteriome plays a protective role against Bd (Rebollar et al., 2020). While we cannot draw cause and effect conclusions about the observed Bd–bacteriome relationships in our observational field study, our results corroborate patterns seen in previous research. If Bd infection is driving the differences exhibited in *R. sierrae* bacteriome communities (Jani et al., 2021; Jani & Briggs, 2014), then our data suggest that Bd infection has little to no influence on the skin bacteriome of *P. regilla*, perhaps indicating that their skin bacteriome is more stable.

Although our study included 23 field sites, we acknowledge that our data are limited to a single point in time. Further studies with multiple sampling events per site can provide a better understanding of consistency and seasonality of infection and bacteriome trends in these co-occurring frogs. It is also important to distinguish between causal mechanisms at different time scales: regardless of whether contemporary tolerance in *P. regilla* is mediated by host or bacteriome, ecological studies such as ours are not designed to identify the past evolutionary drivers of that tolerance. For example, we do not know if past (undocumented) Bd outbreaks in *P. regilla*, or other evolutionary processes, led to the physiological (including microbiota) or life history traits that enable Bd tolerance. Our finding that sympatric species exhibit different disease outcomes and bacteriomes, despite similar pathogen loads, highlights the need for future work to experimentally test the causal links driving these relationships.

CONCLUSIONS

Currently, Bd in the Sierra Nevada is driving dramatic declines of *R. sierrae* and *R. muscosa*, while contemporary *P. regilla* populations appear stable across the same landscape. Our study shows that Bd infection prevalence and infection intensity are similar in MYL frogs and *P. regilla*, suggesting that *P. regilla* tolerates infection, rather than resisting it. We also found that bacteriomes of *P. regilla* and MYL frogs differed, despite these amphibians' co-occurrence within the same field sites, which could be due to differences in life history or physiology. Our finding that sympatric species exhibit

different disease outcomes and bacteriomes, despite similar pathogen loads, highlights the need for future work to experimentally test the causal links driving these relationships.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Microbiome data (raw sequence reads) are archived in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under BioProject Accession Number PRJNA1398947: <https://www.ncbi.nlm.nih.gov/sra/PRJNA1398947>. Amphibian infection data and additional metadata (Miller, 2026) are available from Zenodo: <https://doi.org/10.5281/zenodo.18930458>.

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SUPPORTING INFORMATION

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