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Effects of host species and environment on the skin microbiome of Plethodontid salamanders

Carly R. Muletz Wolz[1,2] | Stephanie A. Yarwood[3] | Evan H. Campbell Grant[4] | Robert C. Fleischer[2] | Karen R. Lips[1]

[1]Department of Biology, University of Maryland, College Park, MD, USA

[2]Center for Conservation Genomics, Smithsonian Conservation Biology Institute, National Zoological Park, Washington, DC, USA

[3]Department of Environmental Science & Technology, University of Maryland, College Park, MD, USA

[4]S.O. Conte Anadromous Fish Research Laboratory, United States Geological Survey Patuxent Wildlife Research Center, Turners Falls, MA, USA

Correspondence
Carly R. Muletz Wolz
Email: craemuletz@gmail.com

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Abstract

1. The amphibian skin microbiome is recognized for its role in defence against pathogens, including the deadly fungal pathogen *Batrachochytrium dendrobatidis* (Bd). Yet, we have little understanding of evolutionary and ecological processes that structure these communities, especially for salamanders and closely related species. We investigated patterns in the distribution of bacterial communities on *Plethodon* salamander skin across host species and environments.
2. Quantifying salamander skin microbiome structure contributes to our understanding of how host-associated bacteria are distributed across the landscape, among host species, and their putative relationship with disease.
3. We characterized skin microbiome structure (alpha-diversity, beta-diversity and bacterial operational taxonomic unit [OTU] abundances) using 16S rRNA gene sequencing for co-occurring *Plethodon* salamander species (35 *Plethodon cinereus*, 17 *Plethodon glutinosus*, 10 *Plethodon cylindraceus*) at three localities to differentiate the effects of host species from environmental factors on the microbiome. We sampled the microbiome of *P. cinereus* along an elevational gradient (n = 50, 700–1,000 m a.s.l.) at one locality to determine whether elevation predicts microbiome structure. Finally, we quantified prevalence and abundance of putatively anti-Bd bacteria to determine if Bd-inhibitory bacteria are dominant microbiome members.
4. Co-occurring salamanders had similar microbiome structure, but among sites salamanders had dissimilar microbiome structure for beta-diversity and abundance of 28 bacterial OTUs. We found that alpha-diversity increased with elevation, beta-diversity and the abundance of 17 bacterial OTUs changed with elevation (16 OTUs decreasing, 1 OTU increasing). We detected 11 putatively anti-Bd bacterial OTUs that were present on 90% of salamanders and made up an average relative abundance of 83% (SD ± 8.5) per salamander. All salamanders tested negative for Bd.
5. We conclude that environment is more influential in shaping skin microbiome structure than host differences in these congeneric species, and suggest that environmental characteristics that covary with elevation influence microbiome structure. High prevalence and abundance of anti-Bd bacteria may contribute to low Bd levels in these populations of *Plethodon* salamanders.

KEYWORDS
amphibians, Appalachia, *Batrachochytrium dendrobatidis*, microbiota, *Plethodon*, symbionts

1 | INTRODUCTION

Patterns of host and microbial associations result from evolutionary and ecological processes acting simultaneously on both host and microbe (Herre, Knowlton, Mueller, & Rehner, 1999; McFall-Ngai, 2005). Factors shaping host-associated microbiomes include (i) evolutionary history of the host (Amato et al., 2015; Council et al., 2016; Larsen, Tao, Bullard, & Arias, 2013); (ii) environmental conditions (Rebollar et al., 2016; Schmidt, Smith, Melvin, & Amaral-Zettler, 2015; Sullam et al., 2012); and (iii) host–microbial interactions (McFall-Ngai & Ruby, 1991; Scheuring & Yu, 2012). Understanding how ecological and evolutionary processes contribute to host-associated community assembly remains a challenge (Nemergut et al., 2013), given limited sampling of host taxa, host body regions and environmental habitats (reviewed by Colston & Jackson, 2016).

Evolutionary history of amphibian hosts is generally the strongest predictor of skin microbiome structure, even for co-occurring species (Belden et al., 2015; Kueneman et al., 2014; McKenzie, Bowers, Fierer, Knight, & Lauber, 2012; Walke et al., 2014). This suggests that host-associated bacterial community composition is not merely a reflection of the micro-organisms in a shared environment. Instead, it implies that there are deterministic mechanisms operating that structure the communities (Loudon et al., 2016; Schmidt et al., 2015), such as biological and chemical traits of the host. Host-associated traits such as chemical content of the skin mucus (Conlon, 2011; Rollins-Smith et al., 2002), skin-shedding rate (Meyer, Cramp, Hernando Bernal, & Franklin, 2012), immune genes (Huang et al., 2016) and diet (Antwis et al., 2014) likely select for specific host communities in amphibians. These traits can differ among species because of underlying ecological and evolutionary processes (Amato et al., 2015; Moeller et al., 2014). However, it is unknown if host-associated traits that influence microbiomes are dissimilar among all species. For instance, amphibian skin microbiome studies have found that microbiome patterns differ among species from different orders (Walke et al., 2014), families (Kueneman et al., 2014; McKenzie et al., 2012; Rebollar et al., 2016) and genera (Belden et al., 2015), yet no study has examined congeneric amphibian species. If congeneric species harbour similar microbial communities, this would suggest that host traits that drive skin-associated bacterial composition are similar for closely related species.

Environment is also a strong predictor of amphibian skin microbiome structure, but to a lesser extent than differences among species (Kueneman et al., 2014; Rebollar et al., 2016). Colonization by bacterial symbionts can occur via environmental transmission (Muletz, Myers, Domangue, Herrick, & Harris, 2012), in which unique sets of bacteria are selected from the regional pool of micro-organisms (Fitzpatrick & Allison, 2014; Rebollar et al., 2016; Walke et al., 2014). Together, these findings suggest that local environmental conditions influence the pool of potential bacterial symbionts, but that host traits influence which bacterial taxa colonize and establish on the skin. Environmental characteristics such as salinity (Schmidt et al., 2015; Sullam et al., 2012) and temperature (Lokmer & Wegner, 2015) predict patterns of microbiome structure in other host-associated microbial communities. The environmental characteristics that influence amphibian skin microbiome structure in the wild are largely unknown, with the exception of two aquatic frog species where water surface area and conductivity explained some of the variation (Krynak, Burke, & Benard, 2016; Kueneman et al., 2014). Surveying along environmental gradients may help identify environmental characteristics that shape host-associated microbial communities.

Host–microbial interactions that promote association between host and microbe can also influence host-associated microbiomes (McFall-Ngai & Ruby, 1991; Scheuring & Yu, 2012). For instance, it has been proposed that inter- and intraspecific variation in the amphibian skin microbiome relates to variation in susceptibility to a deadly, globally distributed pathogen, *Batrachochytrium dendrobatidis* (Bd; Flechas et al., 2012; McKenzie et al., 2012; Kueneman et al., 2014; Rebollar et al., 2016). Some bacteria isolated from amphibian skin can inhibit Bd growth in vitro, hereafter referred to as anti-Bd bacteria, likely through the production of inhibitory metabolites (Brucker et al., 2008). Anti-Bd bacteria can reduce amphibian mortality or weight loss associated with Bd infection in laboratory and field experiments (Becker & Harris, 2010; Harris, Lauer, Simon, Banning, & Alford, 2009; Harris et al., 2009; Vredenburg, Briggs, & Harris, 2011). Loudon et al. (2016) found that environmental bacteria with anti-Bd properties are more likely to colonize an amphibian host than those that lack anti-Bd properties, suggesting a functional role that contributes to community membership. If anti-Bd bacteria were involved in resistance to Bd, we would expect these functionally important bacteria to be abundant and prevalent on amphibians rarely found infected with Bd, such as *Plethodon* salamanders (Muletz, Caruso, Fleischer, McDiarmid, & Lips, 2014). At the individual level, theoretical work by Scheuring and Yu (2012) predicted that the microbiome requires a sufficiently high population density of antibiotic-producing bacteria to fend off pathogens. At the population level, a critical fraction of the population may need a protective microbiome to prevent pathogen transmission, thus leading to herd immunity (Anderson & May, 1990). For instance, Woodhams et al. (2007) found that an amphibian population with 86% of individuals harbouring anti-Bd bacteria was correlated with the population persisting with Bd.

We quantified the roles of host evolutionary relatedness, environmental conditions and fungal–bacterial interactions on determining skin microbiome structure (alpha-diversity, beta-diversity and bacterial operational taxonomic unit [OTU] abundances) on three terrestrial woodland salamander species (*Plethodon cinereus*, *Plethodon glutinosus* and *Plethodon cylindraceus*) from the central Appalachian Mountains. We had three main objectives: (i) compare the microbiome of co-occurring species at three localities to test whether host species factors contributed more to microbiome structure than a shared environment; (ii) examine the microbiome of *P. cinereus* along an elevational gradient at one locality to determine whether elevation predicted bacterial community structure; and (iii) quantify the prevalence and abundance of putatively anti-Bd bacteria to determine if anti-Bd bacteria are dominant members of the skin bacterial

community in a group of salamanders rarely infected with Bd (Muletz et al., 2014).

2 | MATERIALS AND METHODS

2.1 | Field sampling

We collected skin swabs from three species of terrestrial, woodland salamanders, *P. cinereus* (*n* = 71), *P. glutinosus* (*n* = 17) and *P. cylindraceus* (*n* = 12), at sites within three localities located along a 497-km stretch in the central Appalachians in spring 2012 (Table 1). We chose the three localities, Catoctin Mountain Park (MP), MD, Shenandoah National Park (NP), VA, and Mt. Rogers National Recreation Area (NRA), VA, because they (i) were within the range of localities where we had previously tested these species for Bd and found <1% Bd prevalence (Muletz et al., 2014); (ii) were within the distribution of the highly abundant species *P. cinereus* and either *P. glutinosus* or its sister species *P. cylindraceus* (Petranka, 1998); and (iii) were exact localities where we identified culturable, anti-Bd bacteria in a previous study from 61 of the same individuals (Muletz-Wolz et al., 2017). At each site, we recorded GPS coordinates and took two measurements of leaf litter depth and of soil pH (Kelway soil tester) that were averaged to give a site value. For each salamander, we recorded substrate temperature, cover object, species, sex and measured their mass and snout-to-vent length (SVL) to quantify salamander body condition.

We collected skin swabs from each salamander. We used a new pair of nitrile powder-free gloves to handle each salamander, and rinsed each salamander twice for 30 s with sterile water to remove transient microbes (Lauer et al. 2007). We placed each individual into a new plastic bag, and swabbed it 20 times (five strokes each: dorsal/ventral sides and front/back limbs) with a MW-113 swab (Medical Wire, UK). We stored the swab in a 1.5-ml tube on ice until returning to the laboratory where samples were stored in a −80°C freezer until analysis.

2.2 | Molecular methods

We conducted pyrosequencing of 16S rRNA gene amplicons on four Roche 454 GS Junior runs to characterize *Plethodon* skin bacterial communities at the Center for Conservation Genomics (National Zoological Park, Washington, DC, USA). We extracted DNA from the skin swabs using MoBio PowerSoil DNA Extraction kit following the manufacturer's protocol, and included a negative extraction control with each set of sample extractions. We amplified the V3-V5 region of the 16S rRNA gene using the universal gene primer set 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 939R (5′-CTTGTGCGG GCCCCCGTCAATTC-3′). We designed fusion primers such that (i) the forward primer contained the 5′ Roche Amplicon Adapter A followed by an 8-bp barcode (Hamady, Walker, Harris, Gold, & Knight, 2008), a CA linker, and the 515F primer sequence, and (ii) the reverse primer contained the 5′ Roche FLX Amplicon Adapter B followed by the 939R primer sequence. We performed duplicate PCR reactions for each sample, including negative extraction controls, and pooled the duplicate reactions in equal volumes following PCR. Each 25-μl PCR assay consisted of 1.25 U of AmpliTaq Gold DNA Polymerase (ThermoFisher), 2.5 μM $MgCl_2$, 200 nM dNTPs, 200 nM reverse primer, 400 nM forward primer and 3 μl DNA template. PCR conditions were 95°C for 7 m, followed by 30 cycles of 95°C for 45 s, 55°C for 30 s, 72°C for 45 s and a final extension (72°C for 7 m). We used SPRI-beads to clean post-PCR products and ran a subset of samples on a Bioanalyzer chip (Agilent Technologies) to confirm removal of small DNA fragments, primers and excess nucleotides. We quantified molecules per sample using qPCR with a KAPA Library Quantification Kit (Kapa Biosystems), and pooled samples in equimolar amounts.

TABLE 1 Summary of locality, site and species information. We provide a descriptive analysis of the bacterial community and the anti-Bd bacterial community for all 100 individuals. We examined how bacterial community structure varied (i) among species and sites for individuals sampled at sites where two *Plethodon* species co-occurred (co-occurring species dataset), and (ii) along an elevational gradient for *P. cinereus* at Shenandoah NP (denoted by ^)

Locality	No. of sites	Elevation (m)	Species	No. salamanders sampled	No. salamanders co-occurring dataset	Bd prevalence (95% CI per locality)
Catoctin	1	404	*P. cinereus*	7	7	0 (0–22)
Catoctin	1	404	*P. glutinosus*	7	7	
Shenandoah	2	700 ± 3	*P. cinereus*	16^	9	0 (0–6)
Shenandoah	3	797 ± 6	*P. cinereus*	12^	–	
Shenandoah	3	881 ± 18	*P. cinereus*	11^	5	
Shenandoah	2	979 ± 5	*P. cinereus*	11^	–	
Shenandoah	4	697–974	*P. cylindraceus*	7	5	
Mt. Rogers	2	997, 1,053	*P. cinereus*	14	14	0 (0–11)
Mt. Rogers	1	997	*P. glutinosus*	10	10	
Mt. Rogers	1	1,053	*P. cylindraceus*	5	5	
			Total	100	62	

We tested all salamanders for Bd using qPCR. We used primers developed by Boyle, Boyle, Olsen, Morgan, and Hyatt (2004) and iTaq supermix with Rox (Bio-Rad) following their qPCR reaction protocol. We ran all DNA samples in duplicate and used standards of 100, 10, 1, 0.1 zoospore genomic equivalents (ZGEs) developed from Puerto Rican Bd isolate, JEL 427. If one of the duplicates returned a positive signal it was run a third time. Samples were considered positive if they amplified twice before 0.1 ZGEs.

2.3 | Sequence analysis

We used MacQIIME 1.9.1 (Caporaso et al., 2010) and UPARSE (Edgar, 2013) to process the 454 reads. In QIIME, we assigned reads to samples based on their 8-bp barcode, discarded low-quality reads using default parameters, and removed primers. In UPARSE (implemented in USEARCH v7.0), we truncated sequences at positions where quality score were <15 based on Edgar (2013), and then clustered high-quality DNA sequences at 97% similarity, chose a representative sequences for each bacterial OTU and performed chimera checking. Then in QIIME, we used RDP classifier (Wang, Garrity, Tiedje, & Cole, 2007) to assign taxonomy with <80% confidence for each representative OTU using the Greengenes database (May 2013 version). We aligned sequences using PyNAST (Caporaso et al., 2010) and built a phylogenetic tree from the representative sequences using FastTree (Price, Dehal, & Arkin, 2010). We included an archaeal 16S rRNA sequence obtained from Greengenes (GenBank accession #M21087) to root the phylogenetic tree, and trimmed the outgroup using the package APE in R (Paradis, Claude, & Strimmer, 2004). OTUs that failed alignment were removed from downstream analyses. We removed one OTU assigned to the family Enterobacteriaceae that was present in the negative extraction control samples. We used QIIME, to identify the core microbiome, defined as OTUs that were present on 90% or more of individuals in total (Loudon et al., 2014).

2.4 | Measures of microbiome structure

We provide a general descriptive analysis of the bacterial OTUs identified from the three host species (Table 1), including description of their core microbiome. Then, we examined microbiome structure in two subsets of the data: a co-occurring species dataset and an elevational dataset (Table 1; see statistical analyses section). We used three indices to examine microbiome structure: alpha-diversity, beta-diversity and bacterial OTU abundance. We examined alpha- and beta-diversity using both a standard metric and a phylogenetic metric. For alpha-diversity we used total number of observed OTUs (OTU richness) and Faith's Phylogenetic Diversity (Faith's PD: Faith, 1992). For beta-diversity we used Jaccard and unweighted Unifrac (Lozupone, Lladser, Knights, Stombaugh, & Knight, 2011), and we also performed abundance-weighted analyses using Bray–Curtis distances from normalized sequence counts. To generate normalized sequence counts, we performed variance-stabilizing normalization on the raw sequence counts (Paulson, Stine, Bravo, & Pop, 2013) using the functions *cumNormStatFast* and *cumNorm* in the package METAGENOMESEQ (Paulson, Talukder, Pop, & Bravo, 2015). This normalization method corrects for biases associated with uneven sequencing depth (McMurdie & Holmes, 2014; Paulson et al., 2013). For bacterial OTU abundance, we interpreted normalized sequence counts as bacterial abundance because sequence counts are approximately quantitative for microbial OTU abundance (Amend, Seifert, & Bruns, 2010). Hereafter, we refer to normalized sequence counts as bacterial abundance. In bacterial abundance analyses, we filtered the data to contain OTUs that occurred in 5% of samples to reduce spurious significance from OTUs with low sequence counts, and reported false discovery rate (FDR) corrected *p*-values.

We identified OTUs in our dataset that were taxonomically similar (≥97% sequence similarity) to known anti-Bd bacteria (Woodhams et al., 2015), using a custom blast in Geneious 8.1 (Kearse et al., 2012). We queried a database consisting of sequences of anti-Bd bacteria that we identified from 61/100 same individuals from the same *Plethodon* populations (Muletz-Wolz et al., 2017, GenBank accession no. KU738912-KU739030) and the Woodhams et al. (2015) dataset, which consists of anti-Bd bacteria identified from 36 amphibian species sampled globally. We used a megablast program, having Geneious return results as query-centric alignment only and returning only the top hit.

2.5 | Statistical analyses

All statistical analyses were performed in R version 3.2.3 (R Core Team, 2015).

We quantified bacterial community structure at sites in which we sampled at least two salamander species (Table 1: co-occurring species dataset) to determine the relative contribution of host species vs. shared environment in shaping skin microbiomes of sympatric species. For alpha-diversity, we used generalized linear models (GLM) to examine variation in (i) total number of observed OTUs using a quasipoisson distribution to account for overdispersed count data and (ii) Faith's PD using a log-transformation to achieve normality. We included species and site as the main explanatory variables, in addition to covariates of host (sex, body condition, cover object, substrate temperature) and site (leaf litter depth, soil pH). We performed deviance goodness-of-fit tests for each model to assess model fit, and we determined significance of variables using the ANOVA function with χ^2 as the test statistic. For beta-diversity, we used PERMANOVAs (Anderson, 2001) in the package VEGAN (Oksanen et al., 2015) and included the same categorical explanatory variables as in the alpha-diversity analysis. We performed post-hoc analyses using PERMANOVAs, and corrected *p*-values for multiple comparisons using FDR corrections. For quantitative measurements (body condition, soil pH, substrate temperature and leaf litter depth), we examined the effects of these environmental factors on beta-diversity using distance-based linear modelling (function *capscale* in the package VEGAN) with stepwise AIC (Kueneman et al., 2014). We used principle coordinate analysis (PCoA) to visualize beta-diversity patterns using the PHYLOSEQ package (McMurdie & Holmes, 2013). For bacterial abundance, we determined if OTUs

were differentially abundant between species and between sites using zero-inflated log-normal (ZILN) mixture models (Paulson et al., 2013) in the package METAGENOMESEQ (*fitFeatureModel* function; Paulson et al., 2015) with the normalized sequence counts as the response variable. The ZILN mixture models remove testing biases associated with undersampling (Paulson et al., 2013). If sites within a locality had no OTUs that were differentially abundant then we pooled the sites together at the locality level to increase statistical power.

We compared bacterial community structure among allopatric populations of *P. cinereus* sampled along the elevational gradient in Shenandoah NP (Table 1; elevational dataset). For alpha-diversity, we used GLMs to examine variation in total number of observed OTUs and Faith's PD using quasipoisson and Gaussian distributions respectively. We included elevation as the explanatory variable in addition to covariates of host (sex, body condition, cover object, substrate temperature) and site (leaf litter depth, soil pH) characteristics and assessed model fit and significance of variables as above. For beta-diversity, we computed partial Mantel correlations between compositional dissimilarity matrices (Jaccard and Unifrac) and an elevational distance matrix after accounting for spatial distance between sites using 10,000 permutations in the package VEGAN (Oksanen et al., 2015). To examine the effects of environment on beta-diversity, we used distance-based linear modelling with stepwise AIC as above on quantitative measurements (body condition, soil pH, substrate temperature and leaf litter depth). For bacterial abundance, we determined if bacterial OTU abundance was correlated with elevation by using a linear model for each OTU (Jani & Briggs, 2014) with a log-transformation of normalized sequence counts to achieve normality; we report FDR-corrected *p*-values. To visualize changes in significant OTUs over elevations we used PHYLOSEQ and GGPLOT2 packages (Wickham, 2009).

We examined microbiome structure for anti-Bd bacteria as we did above for the entire bacterial community among species, sites and elevations. For alpha- and beta-diversity, we followed the same statistical framework. For abundances of anti-Bd bacterial OTUs we denote those OTUs with an asterisk in tables that report the statistical results from the entire bacterial community analyses.

3 | RESULTS

3.1 | Overview of OTU diversity and core microbiome

We generated 224,503 high-quality bacterial sequences (342 bp average length) from 100 *Plethodon* salamander skin samples representing 480 OTUs from 20 described bacterial phyla. The taxonomic composition of OTUs consisted predominately of bacteria in six phyla (Proteobacteria: $n = 198$, Bacteroidetes: $n = 83$, Actinobacteria: $n = 76$, Firmicutes: $n = 30$, Acidobacteria: $n = 41$, Planctomycetes: $n = 13$).

Most bacterial OTUs (377/480) were rare; they were found on fewer than five individuals, and together made up 2% of total bacterial sequences. Some OTUs were common, namely 12 OTUs, and were found on 90% of individuals (i.e. core microbiome; Table 2), and together made up 88% of total bacterial sequences. Some taxonomic groups were abundant, such as Proteobacteria that had an average relative abundance of 87% per individual (*SD* ± 7) and Actinobacteria representing an average relative abundance of 10% (*SD* ± 5). Most notable were *Acinetobacter* and *Pseudomonas* within the phylum Proteobacteria, as they were widely distributed across species, sites and localities (Table 2) and dominant in abundance (*Acinetobacter*, average = 44%, *SD* ± 18, total OTUs = 5; *Pseudomonas*, average = 32%, *SD* ± 21, total OTUs = 7).

TABLE 2 Core microbiome present on 90% of the salamanders sampled. Asterisks denote OTUs that were taxonomically similar to known anti-Bd bacteria

OTU ID	Phylum	Family	Genus	Avg. relative abundance (%)	Standard error (%)	Range (%)
OTU_1*	Proteobacteria	Moraxellaceae	*Acinetobacter*	32.83	1.77	4–77
OTU_5*	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	15.55	2.08	0–68
OTU_268*	Proteobacteria	Moraxellaceae	*Acinetobacter*	10.07	1.17	0.5–48
OTU_2*	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	9.51	0.82	0.2–28
OTU_3	Actinobacteria	Sanguibacteraceae	*Sanguibacter*	5.4	0.44	0.07–22
OTU_25*	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	4.92	0.68	0–38
OTU_4*	Proteobacteria	Xanthomonadaceae	*Stenotrophomonas*	4.83	0.32	0.4–13
OTU_96*	Proteobacteria	Pseudomonadaceae	–	1.77	0.21	0–11.5
OTU_7*	Actinobacteria	Microbacteriaceae	*Microbacterium*	1.01	0.05	0–2.3
OTU_37*	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	0.94	0.16	0–15.1
OTU_38*	Proteobacteria	Xanthomonadaceae	–	0.79	0.09	0–4
OTU_14*	Proteobacteria	Enterobacteriaceae	–	0.32	0.03	0–1.8
			Total	88		

3.2 | Co-occurring salamanders harboured similar microbiome structure, but composition differed among sites

Bacterial alpha-diversity was similar for all host species at all sites, whereas beta-diversity and bacterial OTU abundances differed among sites, but not between co-occurring species. On average, bacterial alpha-diversity was 59 OTUs per salamander (*SD* ± 18) and a Faith's PD of 4.4 (*SD* ± 1.3), regardless of species or site (Figure 1). None of the host and site covariates tested were related to alpha-diversity (GLM: $p > .05$), indicating high homogeneity in alpha-diversity among salamanders from multiple species and sites. In contrast, we found support for a site effect on beta-diversity: skin microbiomes from individuals at Shenandoah NP were consistently different in bacterial composition compared to those at Catoctin MP and Mt. Rogers NRA (Jaccard, Unifrac and Bray–Curtis, PERMANOVA: $p < .05$), regardless of host species (Figure 2; PERMANOVA: $p > .05$). Soil pH (range 4–6.5) and leaf litter depth (range 1.5–4.1 mm) were the significant factors associated with changes in abundance-weighted bacterial composition (Bray–Curtis, distance-based linear model: $p < .05$), explaining 51% of overall variation. Soil pH was the significant factor correlated with presence–absence compositional changes (Jaccard and Unifrac), and it explained 13% of overall variation. Bacterial abundance varied among localities for 28 OTUs and among species for 5 OTUs (Table 2). All 28 OTUs were differentially abundant between sites at Catoctin MP and Mt. Rogers NRA compared to sites at Shenandoah NP (Table S1, ZILN mixture model: $p < .05$). We did not find any differences in OTU abundances between Catoctin MP and Mt. Rogers NRA (Table S1, ZILN mixture model: $p > .05$). Five of the 28 OTUs were differentially abundant among salamander species (Table S1). However, this was primarily driven by the differences among localities, not within localities, suggesting that co-occurring species generally had similar bacterial abundances (Table 3 and Table S1).

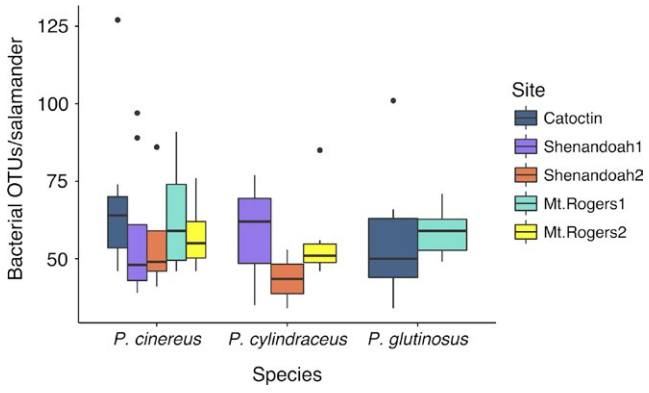

FIGURE 1 Boxplot of total number of operational taxonomic units (OTUs) per individual among three *Plethodon* species sampled at three localities. There was no difference in alpha-diversity metrics among salamander species or sites (OTU richness and Faith's PD; LMM: $p > .05$)

3.3 | Elevation influenced *P. cinereus* skin microbiome composition

Alpha-diversity, beta-diversity and the abundances of 17 bacterial OTUs were correlated with elevation. Alpha-diversity increased with elevation (Figure 3, GLM: OTU richness, $p = .014$, Faith's PD, $p = .004$). For beta-diversity, we found a relationship with elevation, after accounting for distances among sites (Figure 4, partial Mantel: Jaccard, $p = .035$, $R^2 = 9\%$; Unifrac $p = .012$, $R^2 = 11\%$; Bray–Curtis $p = .05$, $R^2 = 6\%$). Soil pH (range 4.8–6.3) and substrate temperature (range 7.5–18.9°C) were the significant factors associated with changes in abundance-weighted bacterial composition (Bray–Curtis, distance-based linear model: $p < .05$), explaining 18% of overall variation. Soil pH was the significant factor correlated with changes in the presence–absence composition (Jaccard and Unifrac) across the gradient, explaining 5% of overall variation. The abundances of 17 bacterial OTUs were correlated with elevation (linear models: $p < .05$, $R^2 = 13\%–43\%$; Table S2), with 16 OTUs decreasing and 1 OTU increasing in abundance with elevation (Figure 5).

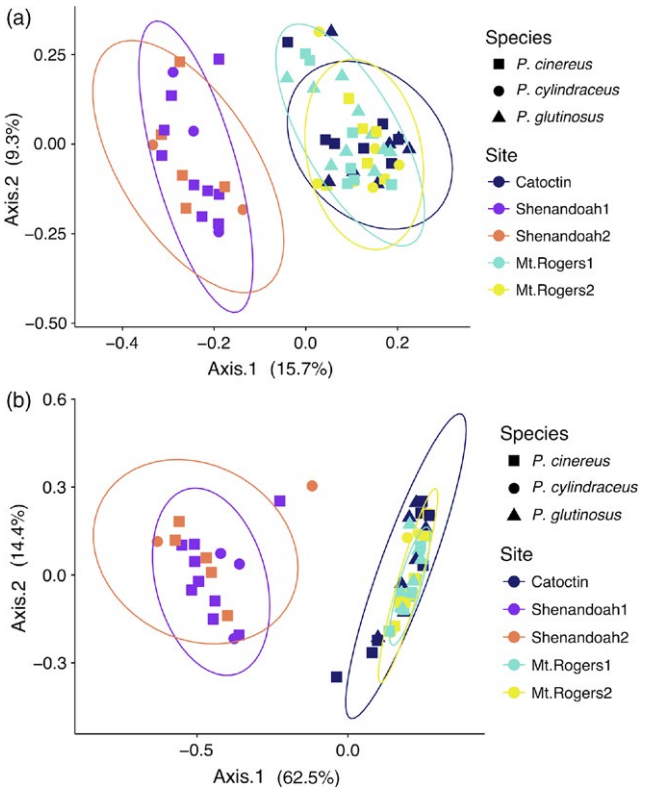

FIGURE 2 Principle coordinate analyses of skin bacterial beta-diversity on three *Plethodon* species sampled at three localities. Sites at Shenandoah NP had significantly different beta-diversity patterns (a: Jaccard distances; b: Bray–Curtis distances) than the sites at Catoctin MP and Mt. Rogers NRA, regardless of salamander species. More variation was explained in the first principal coordinate with the abundance-weighted measure, Bray–Curtis, than by the presence/absence metrics, Jaccard and Unifrac (data not shown), indicating greater differences among sites in abundances of bacterial operational taxonomic units (OTUs) than the presence/absence of OTUs

TABLE 3 Heatmap of the average relative abundance of bacterial OTUs that were differentially abundant among localities. All 28 OTUs were differentially abundant between salamanders at Shenandoah NP compared to salamanders at Mt. Rogers NRA and/or Catoctin MP (ZILN mixture model: $p < .05$). Five OTUs were differentially abundant among salamander species (highlighted in bold: ZILN mixture model: $p < .05$). Asterisks denote OTUs that were taxonomically similar to known anti-Bd bacteria. The use of average relative abundance is intended for interpretation purposes only, and was calculated by dividing each OTU's normalized sequence count by the total normalized sequence count per salamander and then taking the average of that for each species-locality pair (e.g. *Plethodon cinereus* at Catoctin). Formal hypothesis tests were performed using normalized sequence counts as detailed in Materials and methods

OTU ID	Taxa	Catoctin *P. cinereus* (*n* = 7)	Catoctin *P. glutinosus* (*n* = 7)	Shenandoah *P. cinereus* (*n* = 14)	Shenandoah *P. cylindraceus* (*n* = 5)	Mt. Rogers *P. cinereus* (*n* = 14)	Mt. Rogers *P. cylindraceus* (*n* = 5)	Mt. Rogers *P. glutinosus* (*n* = 10)
OTU_2*	*Pseudomonas* sp.	17.71	18.31	1.95	1.81	20.05	14.78	17.85
OTU_3	*Sanguibacter* sp.	8.68	7.03	1.22	0.95	9.71	8.30	7.45
OTU_4*	*Stenotrophomonas* sp.	7.90	8.96	1.81	1.44	8.23	5.78	7.41
OTU_8	f__Cellulomonadaceae	1.14	0.97	0.17	0.14	1.36	1.78	0.90
OTU_25*	*Pseudomonas* sp.	0.72	0.82	8.64	6.81	0.43	0.35	0.40
OTU_5*	*Pseudomonas* sp.	0.64	0.76	50.41	48.71	1.04	0.89	1.10
OTU_165	f__Microbacteriaceae	0.33	0.21	0.04	0.01	0.35	0.23	0.29
OTU_13	*Alicyclobacillus* sp.	0.25	0.14	0.10	0.09	0.16	0.25	0.24
OTU_19*	*Arthrobacter* sp.	0.23	0.10	0.02	0.02	0.16	0.20	0.31
OTU_12	o__Actinomycetales	0.21	0.65	0.01	0.00	0.00	0.01	0.10
OTU_16	*Ochrobactrum* sp.	0.21	0.19	0.05	0.06	0.20	0.18	0.18
OTU_17	*Sphingobium* sp.	0.21	0.10	0.09	0.08	0.15	0.18	0.17
OTU_59*	f__Comamonadaceae	0.18	0.10	0.07	0.02	0.07	0.08	0.07
OTU_11*	*Delftia* sp.	0.11	0.06	0.74	0.42	0.06	0.12	0.03
OTU_34*	*Agrobacterium* sp.	0.06	0.07	0.02	0.01	0.09	0.07	0.07
OTU_38*	f__Xanthomonadaceae	0.05	0.07	1.57	1.08	0.27	0.15	0.33
OTU_50	*Streptococcus* sp.	0.05	0.08	0.00	0.01	0.05	0.03	0.10
OTU_249*	*Pseudomonas* sp.	0.04	0.02	0.58	0.71	0.09	0.03	0.08
OTU_485*	*Stenotrophomonas* sp.	0.04	0.05	0.47	0.10	0.07	0.07	0.08
OTU_158*	*Pseudomonas* sp.	0.03	0.03	0.18	0.14	0.01	0.01	0.02
OTU_79*	*Pseudomonas* sp.	0.03	0.02	0.62	0.77	0.10	0.08	0.05
OTU_275	*Cellulomonas* sp.	0.01	0.03	0.58	0.44	0.05	0.02	0.02
OTU_332	f__Acidobacteriaceae	0.01	0.00	0.01	0.20	0.00	0.01	0.00
OTU_449*	*Pseudomonas* sp.	0.01	0.01	1.35	1.04	0.02	0.02	0.00
OTU_54	f__Methylocystaceae	0.01	0.02	0.07	0.09	0.06	0.05	0.19
OTU_112*	f__Microbacteriaceae	0.00	0.00	0.17	0.08	0.00	0.01	0.00
OTU_148	o__Chlorophyta	0.00	0.04	0.03	0.11	0.01	0.01	0.00
OTU_425*	*Curtobacterium* sp.	0.00	0.00	0.62	0.44	0.06	0.06	0.04

The colours represent the degree of abundance, with warming colours indicating higher abundances.

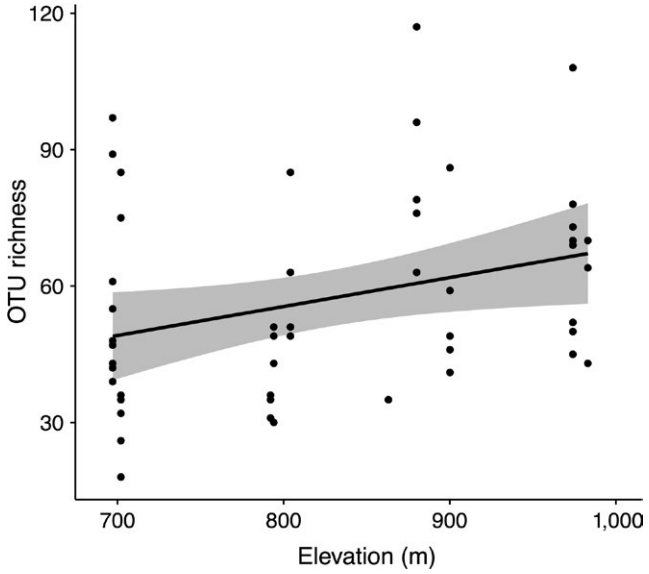

FIGURE 3 Relationship between elevation and alpha-diversity for *Plethodon cinereus*. Bacterial operational taxonomic unit richness increased with elevation (GLM, p = .014)

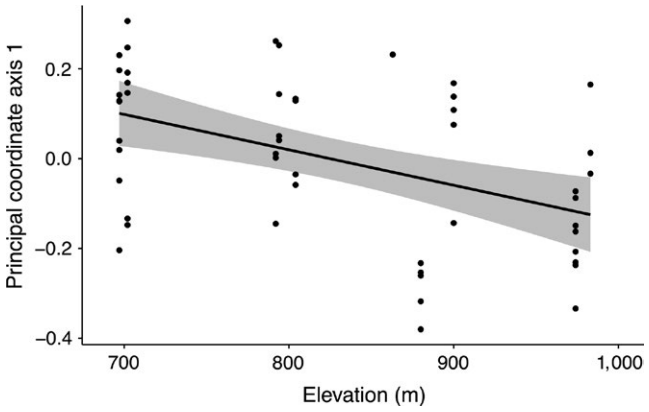

FIGURE 4 Relationship between elevation and principal coordinate axis 1 from Jaccard distances for *Plethodon cinereus*. Bacterial community composition (beta-diversity) changed along the elevational gradient (Partial Mantel tests, p = .035)

3.4 | Anti-Bd OTUs dominated *Plethodon* skin microbiomes and had similar distributional patterns as the entire bacterial community

We found 64 putatively anti-Bd bacterial OTUs in our dataset, making up an average relative abundance of 87% (*SD* ± 8.5) per individual. Across individuals, there was an average of 27 anti-Bd bacterial OTUs per salamander (*SD* ± 4). The same four anti-Bd bacterial OTUs were found on every individual. The core microbiome (defined as OTUs that were present on 90% or more of total individuals; Table 2) consisted of 11 anti-Bd bacterial OTUs that were also dominant in abundance (avg. relative abundance = 83%, *SD* ± 8.5).

Anti-Bd bacterial community structure followed the same patterns in alpha- and beta-diversity as the entire bacterial community across species, site and elevations, except that beta-diversity on *P. cinereus* generally did not show a trend with elevation. For the co-occurring species sampled across multiple sites, alpha-diversity was similar among species and sites with an average of 27 anti-Bd bacterial OTUs per salamander (*SD* ± 4), and anti-Bd bacterial beta-diversity showed differences of individuals at Shenandoah NP being different in bacterial composition compared to those at Catoctin MP and Mt. Rogers NRA (Jaccard, Unifrac and Bray–Curtis; PERMANOVA: p < .05). For *P. cinereus* sampled along the elevational gradient, anti-Bd bacterial alpha-diversity increased with elevation (GLM: OTU richness, p = .013, Faith's PD, p = .004), whereas anti-Bd bacterial beta-diversity changed with elevation for the Unifrac and Bray–Curtis measures (partial Mantel: p = .006, R^2 = 10%; p = .004, R^2 = 12%), but not for the Jaccard metric (partial Mantel: p = .01). Anti-Bd bacterial OTUs that changed in abundance among species, sites and elevations are noted in Table 3 and Table S2.

4 | DISCUSSION

We found that co-occurring *Plethodon* species harboured similar bacterial communities. These data imply that either microbiome composition is only a reflection of the bacteria in the environment or host properties that influence microbiome structure are similar among these closely related host species. It is unlikely that host microbiomes are only a reflection of the environmental pool as empirical evidence from multiple vertebrate host taxa has demonstrated that host-associated bacterial communities are a unique subset from the environmental bacterial communities (Bik et al., 2016; Rebollar et al., 2016; Schmidt et al., 2015; Sullam et al., 2012; Walke et al., 2014) and that host species is generally a strong predictor of bacterial community structure (Amato et al., 2015; Belden et al., 2015; Council et al., 2016; Kueneman et al., 2014; Larsen et al., 2013; Sanders et al., 2015). This suggests that host traits select for unique sets of bacteria from the regional pool of environmental micro-organisms, and that in most species sampled to date these traits are different among species. We hypothesize the main difference between our study and others is that *Plethodon* host factors related to selection for bacterial communities are similar among these closely related species. Factors that likely influence skin microbiomes include antimicrobial peptide composition (Conlon, 2011; Rollins-Smith et al., 2002), skin-shedding rate (Meyer et al., 2012), immune genes (Huang et al., 2016) and diet (Antwis et al., 2014), and are important ecological and evolutionary factors to examine for future work.

We provide evidence that environment is influential in structuring the skin microbiome as community composition varied among sites. Co-occurring species at Shenandoah NP harboured dissimilar bacterial communities from those at Catoctin MP and Mt. Rogers NRA. In geographic distance, Catoctin MP and Mt. Rogers NRA are most distant from one another, and it is plausible that environmental conditions may drive microbiome similarity more so than geographic distance (Sunagawa et al., 2015). For terrestrial vertebrates, such as *Plethodon* salamanders, the environmental attributes that structure microbial communities are largely unknown given limited sampling of

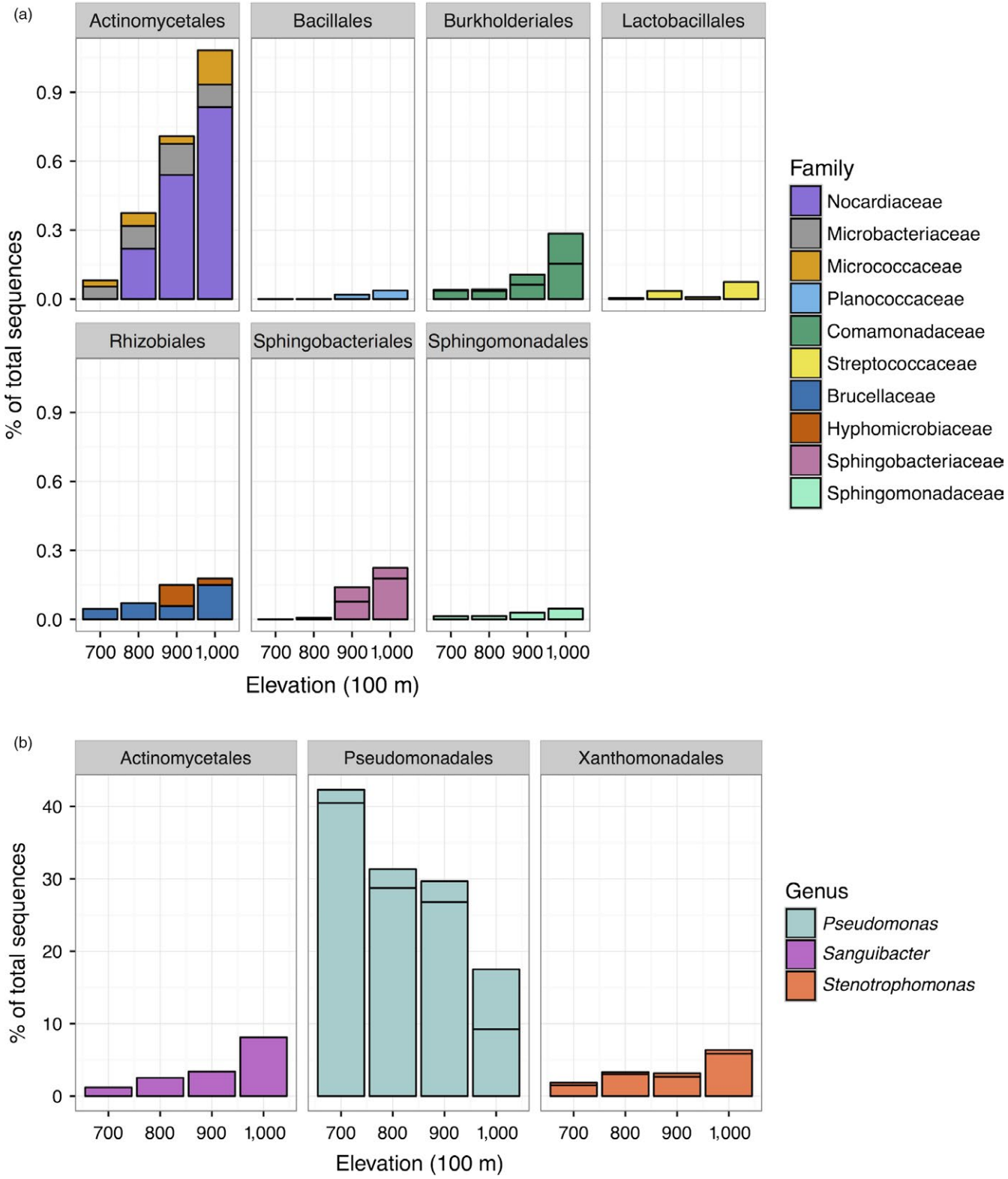

FIGURE 5 Bar plots of operational taxonomic units (OTUs) that showed a correlation between bacterial abundance and elevation (linear model: $p < .05$, R^2 = 13%–43%). Certain bar plots have multiple OTUs that belong to that order and are shown as separate stacks. Twelve OTUs were <1% of the total sequences are shown in (a) at family level taxonomic assignment as not all OTUs could be assigned to genus level. Five OTUs were >1% of total sequences and their lowest taxonomic assignment was genus; they are shown in (b). The use of relative abundance (% of total sequences) on the *y*-axis is intended for visualization purposes only. Formal hypothesis tests were performed using normalized sequence counts as detailed in Materials and methods

wild, terrestrial vertebrates (reviewed by Colston & Jackson, 2016). We found that the environment drove greater changes among sites in bacterial abundances than species presence or absence as environmental characteristics, primarily soil pH, explained more variation in abundance-weighted composition than the presence–absence composition. To better characterize the relationship between environment and bacterial community structure, we sampled *P. cinereus* along an elevational gradient.

Elevation is a complex gradient along which many environmental variables change, and we detected changes in bacterial community structure with elevation, similar to other studies (Bresciano et al., 2015; Lear et al., 2013; Wang et al., 2011). Environment can directly impact bacterial communities by influencing affinity of microbes for substrates, which affects microbial growth (e.g. glutamic acid, phenol, nitrate: Nedwell, 1999; Frey, Lee, Melillo, & Six, 2013) or indirectly by influencing the chemical content of amphibian skin (e.g. AMPs; Krynak et al., 2016) that can regulate the growth of certain microbes (Kueng et al., 2014). The environment can indirectly impact microbiome structure by influencing bacterial species interactions. For instance, Tucker and Fukami (2014) showed that priority effects and temperature alter the abundance of members of a microbial community, such that if certain microbes colonize first they can limit the abundance of other microbes, but this is dependent on temperature. While our study was not designed to disentangle these multiple processes, we do provide evidence that soil pH explained some variation in bacterial composition. Soil pH can drive the spatial distribution of soil bacterial communities (Shen et al., 2013), which may explain the differences in microbiome structure as amphibians can acquire their bacteria environmentally (Muletz et al., 2012).

We demonstrated that the distributional patterns of putatively anti-Bd bacteria across species, sites and elevations were similar to that of the entire bacterial community in our dataset. We do not know the function of all the bacteria present on amphibian skin, since the majority of bacterial species are not cultured (Walke et al., 2015). This provides inference that the community assembly processes of selection, drift, dispersal and speciation events (Vellend, 2010) are acting in a similar way for bacteria of known function and for those in which the function is not known.

Putatively anti-Bd bacteria were prevalent and abundant in the populations sampled, indicating that these symbionts likely serve a functional role for the host and are strong competitors with other microbial taxa. *Plethodon* salamanders encounter a number of potential fungal pathogens in their environment (reviewed in Lauer, Simon, Banning, Lam, & Harris, 2008), and anti-Bd bacteria can produce compounds that are broadly antifungal (Harris, James, Lauer, Simon, & Patel, 2006). Together, both high prevalence and high abundance of anti-Bd bacteria are potential defensive mechanisms at the population- and individual-level against fungal infection, and may be mechanisms that select for certain microbiome structure profiles (Scheuring & Yu, 2012). Loudon et al. (2016) found, in an independent study, that bacteria from the environment are more likely to be overrepresented on *P. cinereus* skin if they have anti-Bd properties; they suggest that selection for anti-Bd bacteria is plausible given the strong selective pressure of Bd and other fungal pathogens, and the century-long existence of Bd in North America (Ouellet, Mikaelian, Pauli, Rodrigue, & Green, 2005; Talley, Muletz, Vredenburg, Fleischer, & Lips, 2015). While we cannot conclude that anti-Bd bacterial dominance is the mechanism of defence against Bd for these salamanders, we do provide evidence that anti-Bd bacteria are dominant members of *Plethodon* salamander skin microbial communities. The protective role these bacteria may offer against the closely related *Batrachochytrium salamandrivorans* that is currently absent in this salamander biodiversity hotspot (Martel et al., 2014) is unknown, but warrants future research.

In conclusion, our results show that host species-specific microbial communities are not always detected and that, in some cases, host factors that select for microbial communities are similar among species. Furthermore, the environment was a strong predictor of skin microbiome structure demonstrating the role of environmental filtering, driven in part by soil pH, in host-associated community assembly. Finally, the dominance of anti-Bd bacteria on *Plethodon* salamanders suggests that these antifungal bacteria may provide a functional role in protection of these salamanders from fungal pathogens. Results from this work contribute to our understanding of how skin-associated bacteria are distributed across the landscape, among host species, and their putative relationship with disease.

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AUTHORS' CONTRIBUTIONS

C.R.M.W. and K.R.L. designed the research. C.R.M.W. conducted the field work with contributions from K.R.L. and E.H.C.G. R.C.F. provided the facilities and equipment to perform the molecular work. C.R.M.W. conducted the molecular work with advice from R.C.F. and S.A.Y. C.R.M.W. analysed the data with advice from E.H.C.G. All authors contributed to the writing of the manuscript.

DATA ACCESSIBILITY

Pyrosequencing runs have been deposited in the National Center for Biotechnology Information Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under accession number SRP107951. Environmental metadata is available from figshare: https://doi.org/10.6084/m9.figshare.5032499.v1 (Muletz Wolz, Lips, Yarwood, Grant, & Fleischer, 2017).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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