Bacterial Skin Assemblages of Sympatric Salamanders are Primarily Shaped by Host Genus

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Abstract

Bacterial assemblages on the skins of amphibians are known to affect pathogen resistance and other important physiological functions in the host. Host-specific factors and the environment play significant roles in structuring skin assemblages. This study used high-throughput 16S rRNA sequencing and multivariate analyses to examine differences in skin-bacterial assemblages from 246 salamanders belonging to three genera in the lungless family Plethodontidae along multiple spatial and temporal gradients. Composition and α- and β-diversity of bacterial assemblages were defined, and the relative influences of host- versus environment-specific ecological factors were evaluated. At the broadest spatial scale, host genus and sampling site were both predictive of skin assemblage structure, but host-specific variation was more influential after controlling for the marginal effects of site, as well as nestedness of site. Furthermore, assemblage similarity within each host genus did not change with increasing geographic distance. At the smallest spatial scale, site-specific climate analyses revealed different relationships to climatic variables for each of the three genera, and these relationships were determined by host ecomode. Variation in bacterial assemblages of terrestrial hosts correlated with terrestrial climatic variability, and this pattern decayed with increasing water dependence. Results from this study highlight host-specific considerations for researchers studying wildlife diseases in co-occurring, yet ecologically divergent, species.

1. Introduction

The vertebrate skin-microbial community functions as part of the innate immune system, providing physical and chemical barriers to pathogens [1–2]. The amphibian skin microbiome is known to protect its host in numerous ways, including antimicrobial peptide induction, direct production of antifungal metabolites, and defense against important pathogens such as *Batrachochytrium dendrobatidis* and *B. salamandrivorans* [3–6]. Defining the composition and assembly processes affecting the amphibian cutaneous microbiome is important for understanding its role in pathogen defense and other physiological functions in the host [4].

Past work using 16S metabarcoding has revealed that skin assemblage composition differs according to host taxon, life history stage, geographic region, microhabitat type, and even body regions within amphibian individuals [7–10]. Landscape-level ecological factors known to shape the amphibian skin-bacterial assemblage include abiotic parameters such as humidity, temperature [9], elevation [11], and microbial reservoirs in soil [12–13]. Environmental dispersal influences skin bacterial assemblages of plethodontid salamanders, but host-specific constituents have also been reported [12–15]. Significant associations between the skin-bacterial assemblage and amphibian host phylogeny have been found at the order [16], family [8–9], genus [17], species, and even subspecies ranks [18]. Conversely, separate studies comparing terrestrial species of lungless salamanders found that selection of skin bacteria is driven primarily by the host's environment and, secondarily, by its taxonomy [15, 19].

Because salamander life history traits inextricably bind them to distinct microhabitats, and thus, potentially unique bacterial reservoirs [20], it is possible that host-specific variation may be more difficult to resolve at lower taxonomic levels due to distinct environmental reservoirs for microbial dispersal. Distance decay is defined as a decrease in assemblage similarity with increasing geographic or environmental distance. Analyses of distance decay, when coupled with an analysis of spatial and climatic data, allow for explicit tests of processes acting on skin assemblage diversity at both the local and regional scales to help disentangle effects of ecological factors and elucidate patterns in community assembly processes [21–23]. This study takes advantage of the overlapping geographic distribution, close proximity of salamander microhabitats at a particular site, and evolutionary relatedness of the genera *Desmognathus, Eurycea*, and *Plethodon* in order to assess potential factors structuring skin-bacterial assemblages of lungless salamanders of the eastern U.S.

The southeastern U.S. hosts the most biodiverse assemblage of amphibian species on planet Earth [24–25]. At least 56 amphibian species are found within the state of Tennessee [26]. Tennessee consists of eight distinct ecoregions (Environmental Protection Agency level III) with different geology, vegetation, and hydrology [26–27]. Three genera of salamanders in the lungless family Plethodontidae (*Desmognathus, Eurycea, Plethodon*) are found ubiquitously across the three easternmost Tennessee ecoregions (Cumberland Plateau, Ridge and Valley, Blue Ridge Mountains). These genera have distinct life-history characteristics and divergent ecomodes. The genus *Desmognathus* is mostly aquatic and usually found in headwater and secondary order streams [26]. The genus *Eurycea* is semiaquatic and often found directly adjacent to streams beneath rocks and logs on the riverbank [26]. The genus *Plethodon* is completely terrestrial and often found just a few meters away from the stream bank beneath moist decaying logs [26]. The sympatric geographic distribution and distinct life histories of these salamander genera make them an ideal study system to understand the relative importance of host characteristics and/or environmental influence in structuring their skin microbiomes.

The objectives of this project were to use high-throughput 16S rRNA metabarcoding and sequencing to (1) characterize the bacterial skin communities of three co-occurring but ecologically divergent genera of plethodontid salamanders across three ecoregions of Tennessee, USA, and two seasons of the year, (2) measure turnover in OTU assemblage structure across two spatial gradients (i.e., physiographic ecoregions and salamander genera) with distance decay analyses, and (3) partition variation in assemblage structure according to geography and host genus. We hypothesized that variation in bacterial assemblages would be explained primarily by host genus, but that degree of variation would increase with increasing geographic distance of a single host.

2. Methods

2.1. Sample Collection

Adult salamanders belonging to the family Plethodontidae were targeted for skin swab sampling in this study, including the genera *Plethodon* (n=74), *Eurycea* (n=65), and *Desmognathus* (n=107). These three

genera co-occur at sampling sites but utilize a diversity of ecomodes (terrestrial, semi-aquatic, and mostly aquatic, respectively). Nine protected sites across three physiographic ecoregions of Tennessee were sampled – the Cumberland Plateau, Ridge and Valley, and Blue Ridge Mountains. Samples were collected during concurrent weeks in both 2016 and 2017, specifically, May 13 – 21 and July 21 – 30, to provide a latitudinal understanding of microbial communities through seasons and calendar years. Members of all three salamander genera were sampled at each site, except for one site within the Ridge and Valley ecoregion where the genus *Plethodon* does not occur.

Each salamander was captured by hand, placed into a clean plastic bag with one corner removed for water drainage, and rinsed for 60 seconds with 2 hour-autoclaved Millipore water to remove transient microbes. A sterile rayon-tipped swab was rolled across all body surfaces for 15 strokes, and stored in an empty, sterile 2mL tube to be used for characterization of cutaneous bacterial assemblages via 16S metabarcoding and high-throughput sequencing. A full-process negative control sample was collected at field sites by rinsing and swabbing the inside of an empty plastic bag (as above), so that contaminating bacteria could be sequenced and removed during bioinformatics processing of sequence data, described below. Salamanders were released the same day at the same location where they were captured. Collection permits were obtained (United States Department of the Interior National Park Service permit no. GRSM-2017-SCI-1263), and institutional guidelines for ethical use of animals in research were observed (IACUC permit no. 15-16-001). All samples were promptly stored in a -12° C portable freezer, transported to the laboratory, and stored at -20° C until processing.

2.2. High-throughput Sequencing and Bioinformatics

Total genomic DNA was extracted from swab tips using the QiaAMP PowerFecal Kit (QIAGEN, Inc., Hilden, Germany). For each DNA extraction, a single sterile swab was processed as above to serve as a negative control (DNA extraction blank). The V4 region of the 16S rRNA gene was PCR-amplified with MC Lab I-5™ 2X Hi-Fi Master Mix (Molecular Cloning Laboratories, South San Francisco, CA) and primers 515F and 806R containing i5 and i7 Illumina flow cell adapter sequences [as in 28]. During amplicon PCR, autoclaved Millipore water was used in place of DNA in a single well of the PCR plate, to serve as an additional negative control sample. Amplicons were dual-indexed with Nextera XT Index Kit Set B, and sequenced on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA) using 2 × 250 bp paired-end reads. Modifications to the Illumina library preparation protocols are described in Table S1.

To characterize bacterial assemblage composition, mothur v. 1.40.3 was used to screen sequences according to standard filtering parameters, cluster sequences at 97% identity into operational taxonomic units (OTUs), assign taxonomy to OTUs, and remove non-target and contaminant sequences [29; Appendix A]. Contaminant OTUs were removed from the dataset including those found in full-process field negatives, DNA extraction blanks, and PCR negative control samples (n=19,846 OTUs). OTUs were used in downstream analyses, rather than ASVs (amplicon sequence variants), as negligible differences have been noted between approaches in ecological studies [30]. The taxonomy of each OTU was determined by comparision to the SILVA v.132 reference database [31]. Final sequence counts for samples were compared after quality control and found to be significantly different across libraries

(Kruskal-Wallis $\chi^2 = 48.462$, df = 3, $p < 0.001$). Therefore, the final dataset was subsampled at 2,009 sequences per sample to account for library size differences [32]. For all downstream statistical analyses described below, OTUs occurring fewer than twice per sample were excluded from that particular sample as it has been shown that rare taxa can influence assessments of ecological communities [33]. The final OTU × sample matrix consisted of 246 skin swab samples from salamanders belonging to the genera *Desmognathus* (n=107), *Eurycea* (n=65), and *Plethodon* (n=74); and 16,381 OTUs (Table 1).

Table 1
Number of skin swab samples collected from salamanders belonging to the genera *Desmognathus*, *Plethodon*, and *Eurycea*, in three physiographic ecoregions of Tennessee, USA.

Ecoregions	*Desmognathus*	*Plethodon*	*Eurycea*
Cumberland Plateau	37	24	13
Ridge and Valley	34	20	27
Blue Ridge Mountains	36	30	25

2.3 Statistical Analyses

We followed the statistical framework defined by Marti Anderson and colleagues [34] and used their dual definitions of β-diversity to (1) measure turnover in OTU assemblage structure across two spatial gradients (i.e., physiographic ecoregions and salamander genera), and (2) partition variation in assemblage structure according to geography and host genus. All analyses were conducted in R ver. 4.0.3 [35]. Shannon and inverse Simpson indices of α-diversity were calculated, and Shapiro-Wilks tests for homogeneity of variance were conducted on these α-diversity metrics. For sample groups with heterogeneous variance, nonparametric Kruskal-Wallis tests were used to determine differences in α-diversity among groups. Sorensen dissimilarity values of total β-diversity were calculated from a matrix of OTU presence/absence data [as in 36], and *betadisper* tests of multivariate dispersion in package *vegan* were conducted on Sorensen indices. To determine if β-diversity is similar among co-occurring salamander genera, differences in dispersion were assessed at each of the nine field sites, and both within and among the three Tennessee ecoregions. Differences in dispersion were compared using principal coordinates analysis (PCoA). To determine the effect of host genus, ecoregion, site, and season on skin microbiome structure, the *adonis* function in package *vegan* was used to conduct permutated multivariate analyses of variance (PERMANOVAs; Bonferonni-corrected $\alpha = 0.0125$). We assessed broad-scale patterns in the skin microbiome by running a PERMANOVA for all samples, and ecoregion-specific patterns using a second set of PERMANOVAs for each ecoregion individually. Analyses were conducted with permutations constrained within sites to account for nestedness of sites within ecoregions (*strata = site*). Distance-based redundancy analyses were conducted *post hoc* to disentangle marginal effects of putatively correlated factors. To visualize differences in bacterial assemblage structure, OTU presence/absence data were plotted by sample type in two-dimensional nonmetric multidimensional scaling (NMDS) ordinations.

To assess the influence of geographic distance, we compared total β-diversity (SOR), turnover (SIM), and nestedness (SNE) components of Sørensen diversity across geographic distances, following the methods of Grisnik et al. [28]. Specifically, we calculated the geographic distances between sample points (calculated as Euclidian distances) using the *dist* function in the *vegan* package. Due to issues with singular fit in the mixed models, we removed nestedness of our data by averaging beta diversity metrics (SOR, SIM, SNE) by the dummy variable "site contrast." The site contrast variable described the pairwise site-level comparisons. We then used a generalized linear model (GLM; function *glm*), to compare average assemblage structure and geographic distance. The GLM assumed a binomial distribution, with average geographic distance and host genera set as fixed effects and a Bonferroni-adjusted p-value of 0.016. To determine if there was a difference in the rate of distance decay across the three genera, we used function *anova* (package *car*) with a Type II sum- of-squares to account for unequal sample sizes across groups.

We acquired climatic data for each site from the Worldclim database (http://www.worldclim.org) at 30 second resolution (1km). These data represent a series of 19 bioclimatic variables derived from global temperature and precipitation grids [37]. Prior to analysis, we selected five bioclimatic variables based on hypothesized importance and removed highly correlated (> 0.75) variables [38]. The five variables included in this analysis were precipitation seasonality (Bio15), mean diurnal temperature range (Bio2), isothermality (day-to-night temperature variability across seasons; Bio3), precipitation of driest quarter (Bio17), and maximum temperature of the warmest month (Bio5). We then extracted the values for each climatic variable using the *extract* function in the *raster* package [39]. To elucidate how climatic variables influenced β-diversity across sites, we compared variation in multivariate dispersion (*betadisper*) across the five selected climatic variables. We used a Bonferroni-adjusted p-value of 0.01 to account for multiple comparisons.

3. Results

An average richness of 591 OTUs (range 3 – 3,601) was recovered per skin swab. The most abundant bacterial phyla shared by all three salamander genera were Proteobacteria, Verrucomicrobia, Acidobacteria, Actinobacteria, and Bacteroidetes (Fig. 1). Shapiro-Wilks tests indicated that data were not normally distributed within salamander genera ($p < 0.05$ for all tests). Both measures of α-diversity differed among salamander genera (Shannon index: Kruskal-Wallis $\chi^2 = 22.752$, $p < 0.001$; inverse-Simpson index: Kruskal-Wallis $\chi^2 = 32.472$, $p < 0.001$).

At all nine sites and for all three ecoregions, multivariate dispersion of bacterial skin assemblages (*betadisper*) did not differ among host genera ($p > 0.05$ for all tests). When the entire dataset was included, the average structure of bacterial assemblages differed only by salamander genus (PERMANOVA $p < 0.001$, $R^2 = 0.0407$, Table 2). There was a significant interaction effect between genus and ecoregion ($p < 0.001$), indicating that host-specific differences in bacterial assemblage structure vary regionally. Pairwise comparisons indicated that skin assemblages were distinct for each genus (Bonferroni-adjusted $p = 0.003$ for each pair of host genera, Fig. 2).

Table 2

PERMANOVA results for main effects tests of the variables Genus, Ecoregion, and Season, and Genus-Ecoregion interaction. Terms were added sequentially (first to last). Abbreviations: df = Degrees of freedom; SS = Sum of Squares; MS = Mean Square; Pr(>F) = percent of permutations resulting in values greater than Pseudo-F.

Source	df	SS	MS	Pseudo-F	R^2	Pr(>F)
Genus	2	4.179	2.08964	5.3134	0.04072	0.001 ***
Ecoregion	2	2.704	1.35189	3.4375	0.02635	0.645
Season	1	0.527	0.52677	1.3394	0.00513	0.033
Genus:Ecoregion	4	2.400	0.60007	1.5258	0.02339	0.001 ***
Residuals	236	92.814	0.39328		0.90441	
Total	245	102.624			1.00000	

PERMANOVAs, as implemented with function *adonis*, add factors sequentially during R^2 calculation, but the sequence of terms matters when factors are correlated and linear dependency can be assumed, as in the case of host genus and site. Distance-based redundancy analyses (function *dbrda*) were required to find marginal effects for these two factors [34]. After controlling for the effect of host genus, the marginal effect of site was still significant, and the inverse was also true (*dbrda*; Genus: $F_{2, 235} = 4.7661$, $p \leq 0.001$; Site: $F_{8, 235} = 2.4884$, $p \leq 0.001$). These analyses were repeated for each ecoregion separately. For all three ecoregions, host genus explained a greater proportion of skin assemblage variation than site, whether permutations were constrained within sites (*adonis*; Cumberland Plateau: Genus: $F_{2, 65} = 2.1565$, $R^2 = 0.05594$, $p \leq 0.001$; Site: $F_{2, 65} = 1.6793$, $R^2 = 0.04356$, $p = 0.072$; Ridge and Valley: Genus: $F_{2, 73} = 2.6833$, $R^2 = 0.06177$, $p \leq 0.001$; Site: $F_{2, 73} = 2.4127$, $R^2 = 0.05554$, $p = 0.981$; Blue Ridge: Genus: $F_{2, 82} = 3.9375$, $R^2 = 0.07914$, $p \leq 0.001$; Site: $F_{2, 82} = 2.2490$, $R^2 = 0.04520$, $p = 0.035$; Table 3) or not constrained within sites (Cumberland Plateau: Genus: $F_{2, 65} = 2.1565$, $R^2 = 0.05594$, $p \leq 0.001$; Site: $F_{2, 65} = 1.6793$, $R^2 = 0.04356$, $p \leq 0.001$; Ridge and Valley: Genus: $F_{2, 73} = 2.6833$, $R^2 = 0.06177$, $p \leq 0.001$; Site: $F_{2, 73} = 2.4127$, $R^2 = 0.05554$, $p \leq 0.001$; Blue Ridge: Genus: $F_{2, 82} = 3.9375$, $R^2 = 0.07914$, $p \leq 0.001$; Site: $F_{2, 82} = 2.2490$, $R^2 = 0.04520$, $p \leq 0.001$).

Table 3

PERMANOVA results for main effects tests of the variables Genus, Site, and Genus-Site interaction within the (a) Cumberland Plateau, (b) Ridge and Valley, and (c) Blue Ridge ecoregions when permutations were constrained within sites. Terms added sequentially (first to last). Abbreviations: Ge = Genus; Res = Residuals; df = Degrees of freedom; SS = Sum of Squares; MS = Mean Square; Pr(>F) = percent of permutations resulting in values greater than Pseudo-F.

(a) Cumberland Plateau

Source	df	SS	MS	Pseudo-F	R^2	Pr(>F)
Ge	2	1.6841	0.84205	2.1565	0.05594	0.001 ***
Site	2	1.3114	0.65572	1.6793	0.04356	0.072
Ge x Site	4	1.7283	0.43207	1.1065	0.05741	0.070
Res	65	25.3805	0.39047		0.84309	
Total	73	30.1043			1.00000	

(b) Ridge and Valley

Source	df	SS	MS	Pseudo-F	R^2	Pr(>F)
Ge	2	2.0670	1.03336	2.6833	0.06177	0.001 ***
Site	2	1.8580	0.92918	2.4127	0.05554	0.981
Ge x Site	3	1.4210	0.47374	1.2301	0.04248	0.015 *
Res	73	28.1113	0.38511		0.84022	
Total	80	33.4600			1.00000	

(c) Blue Ridge

Source	df	SS	MS	Pseudo-F	R^2	Pr(>F)
Ge	2	2.7830	1.39147	3.9375	0.07914	0.001 ***
Site	2	1.5900	0.79478	2.2490	0.04520	0.035 *
Ge x Site	4	1.8130	0.45323	1.2825	0.05156	0.011 *
Res	82	28.978	0.35339		0.82410	
Total	90	35.163			1.00000	

No significant distance-decay relationship was found for any measure of β-diversity (GLM; SOR: $z = 0.002$, $p > 0.05$; SIM: $z = 0.05$, $p > 0.05$, SNE: $z = -0.082$, $p > 0.05$; Fig. 3), and there was no significant difference in the rate of decay between the three genera for all three measures of β-diversity (ANOVA; SOR: $p > 0.05$, SIM: $p > 0.05$, SNE: $p > 0.05$).

For analyses of climate data, total β-diversity (SOR), measured as multivariate dispersion, was significantly different across precipitation seasonality values for all three genera (*betadisper*, *Plethodon*: $F_{3, 70} = 7.6037$, $p \leq 0.01$, *Eurycea*: $F_{3, 61} = 13.899$, $p \leq 0.01$, *Desmognathus*: $F_{3, 100} = 8.4202$, $p \leq 0.01$).

Additionally, β-diversity measured as multivariate dispersion (*betadispr)* varied across values for mean diurnal range for both *Plethodon* and *Eurycea* (*Plethodon*: $F_{6, 67} = 5.4202$, $p \leq 0.01$, *Eurycea*: $F_{7, 57} = 2.2618$, $p > 0.01$), but not for *Desmognathus* microbial assemblages ($F_{7, 96} = 2.1205$, $p > 0.01$). Interestingly, all other climatic variables were only significantly predictive of *Plethodon* salamander microbial assemblages (isothermality: $F_{4, 69} = 4.4332$, $p \leq 0.01$, precipitation of the driest quarter: $F_{7, 66} = 3.7282$, $p \leq 0.01$, maximum temperature of warmest month: $F_{6, 67} = 3.3851$, $p \leq 0.01$), but not for *Eurycea* (isothermality: $F_{5, 59} = 2.3785$, $p > 0.01$, precipitation of the driest quarter: $F_{8, 56} = 1.8114$, $p > 0.01$, maximum temperature of warmest month: $F_{6, 58} = 4.0724$, $p \leq 0.01$) or *Desmognathus* (isothermality: $F_{5, 98} = 2.4632$, $p > 0.01$, precipitation of the driest quarter: $F_{8, 95} = 1.3033$, $p > 0.01$, maximum temperature of warmest month: $F_{6, 97} = 1.9748$, $p > 0.01$).

4. Discussion

The structure of the vertebrate skin-bacterial community depends on environmental, climatic, and host-related factors. This study characterized the salamander cutaneous bacterial assemblage within a fine-resolution ecological framework and elucidated relative influences of these factors. Not surprisingly, both host genus and sampling site were identified as factors that are influential in structuring skin assemblages, and there was a significant interactive effect of host genus and ecoregion. At the broadest geospatial scale, when all samples from all regions were included, it was not possible to disentangle the relative influences of host genus and site on bacterial assemblage structure. Looking within each ecoregion, however, a clearer pattern emerged. At the regional level, there was a stronger effect of host genus on skin β-diversity compared to site. These results, consistent with the findings of Buttimer et al. [17], support the conclusion that the influence of the environment varies by host genus. In other words, host genus likely determines the degree to which the bacterial assemblage is shaped by the environment, and this effect seems to be scale-dependent, with the strength of the environmental signal increasing at smaller spatial scales.

We found no turnover or differences in β-diversity of bacterial skin assemblages, and this lack of a decay for geographic distance indicated that the interaction effect we observed between host genus and ecoregion is not driven by stochasticity (neutral processes), but most likely the environment [21-22]. Site-specific climate analyses provided a more fine-scale view of the role of the environment in shaping skin assemblages and further reinforced the conclusion that host-related factors and the environment are interacting to shape the skin assemblage. Within our system, a climatic gradient was observed in *Plethodon* (terrestrial) assemblages and, to some extent, *Eurycea* (semi-terrestrial) assemblages, but not in *Desmognathus* (aquatic) assemblages. Therefore, the observed pattern of climate-driven variation decayed with increasing water dependence of the host, and this may be explained by the fact that aquatic microhabitats are buffered from terrestrial climatic variability. Seasonal temperature fluctuations, for example, are relatively milder in aquatic microhabitats and may have less of an effect on bacterial assemblages of stream-dwelling salamanders. Conversely, terrestrial salamanders of the genus

Plethodon are less capable of seeking out cooler microclimates, so their skin assemblages likely covary with temperature.

With the impending arrival of the chytrid pathogen *Batrachochytrium salamandrivorans*, which threatens rare and endemic salamander populations in North America [40], researchers continue to uncover ecological assembly processes of the cutaneous microbiome. This study provides further evidence that the skin-bacterial assembly is shaped by host-related factors. This is important because susceptibility to infection is known to vary by host species [41-42]. For example, chytridiomycosis is known to be lethal in *Eurycea wilderae* and *Plethodon metcalfi* [41, 43], but the sympatric species *Desmognathus monticola* seems more resistant [42]. We found that *Desmognathus* salamanders harbor bacterial assemblages that are relatively resilient against landscape-level climatic variation. Future work should investigate whether resilience of the bacterial assemblage against changes in microclimate facilitates pathogen defense, for it is the structural stability of the cutaneous microbiome, rather than species composition, which likely governs its immune function, and consequently, manifestation of disease in the host [10, 44].

Declarations

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Author Contributions:

The three authors contributed to all aspects of conceptualization, methodology, formal analysis, investigation, and data curation. AJH wrote the manuscript and all authors contributed to revisions. All authors have agreed to the published version of the manuscript.

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Figures

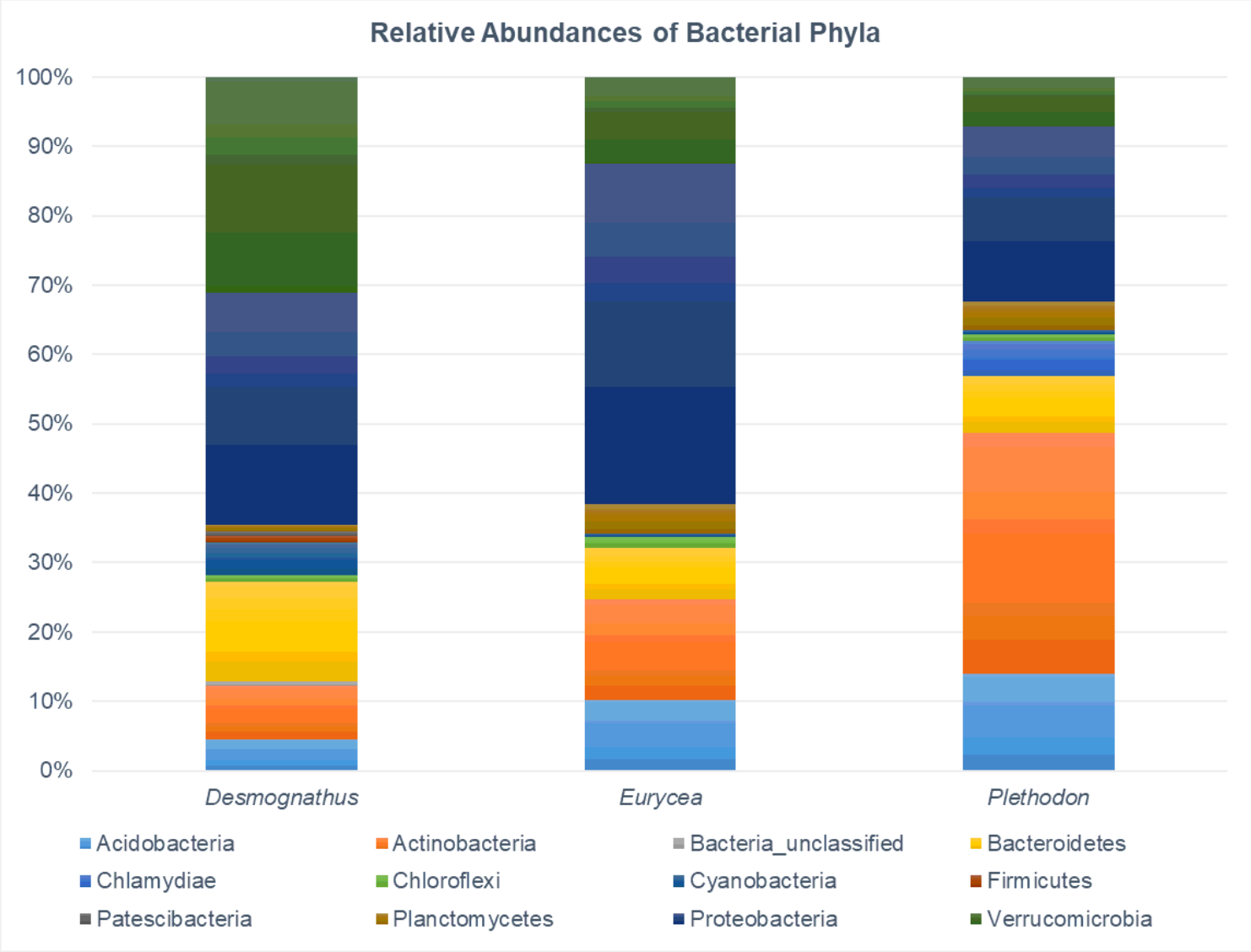

Figure 1

Most abundant bacterial phyla in complete skin-bacterial assemblages of salamanders belonging to the genera *Desmognathus*, *Eurycea*, and *Plethodon*

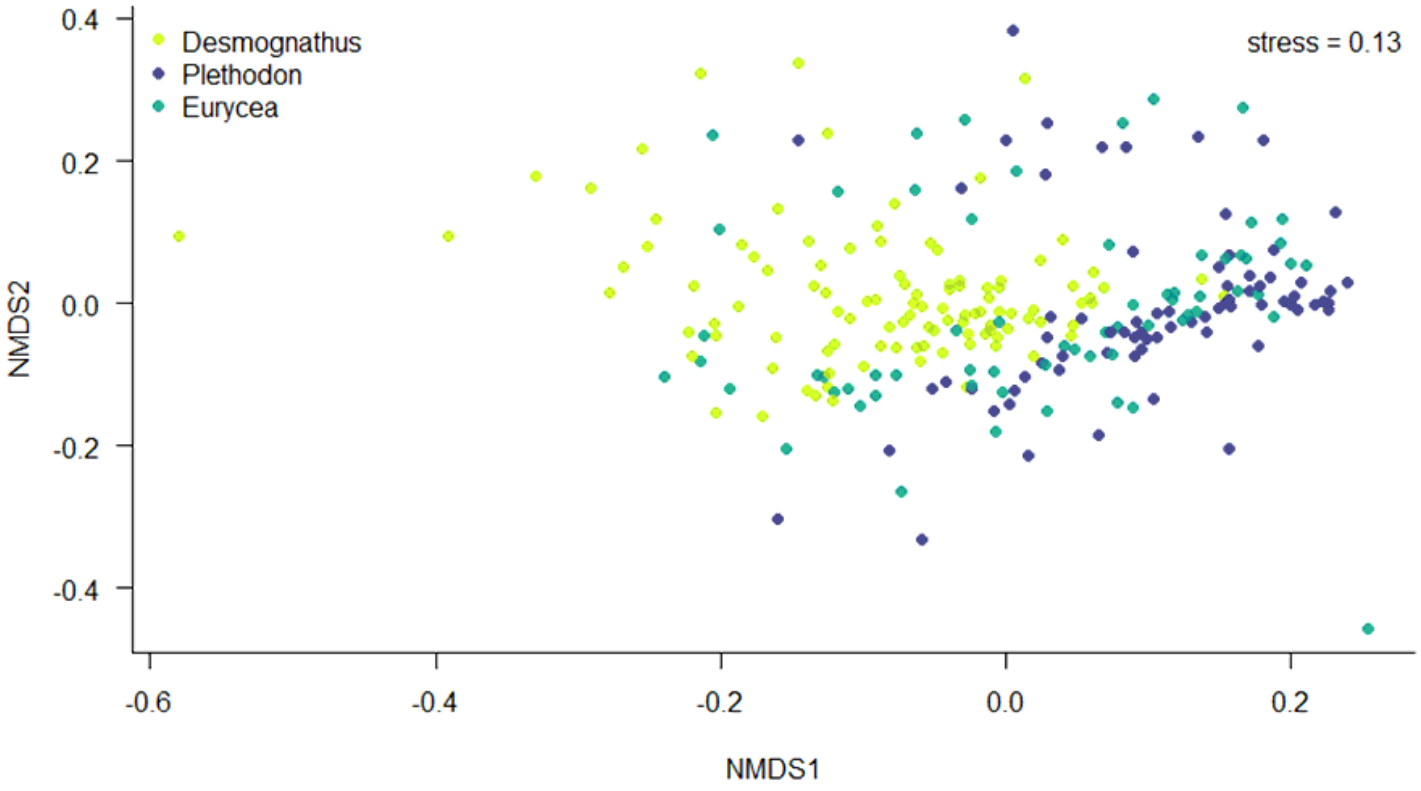

Figure 2

Two-dimensional NMDS ordination of skin communities plotted by salamander genus

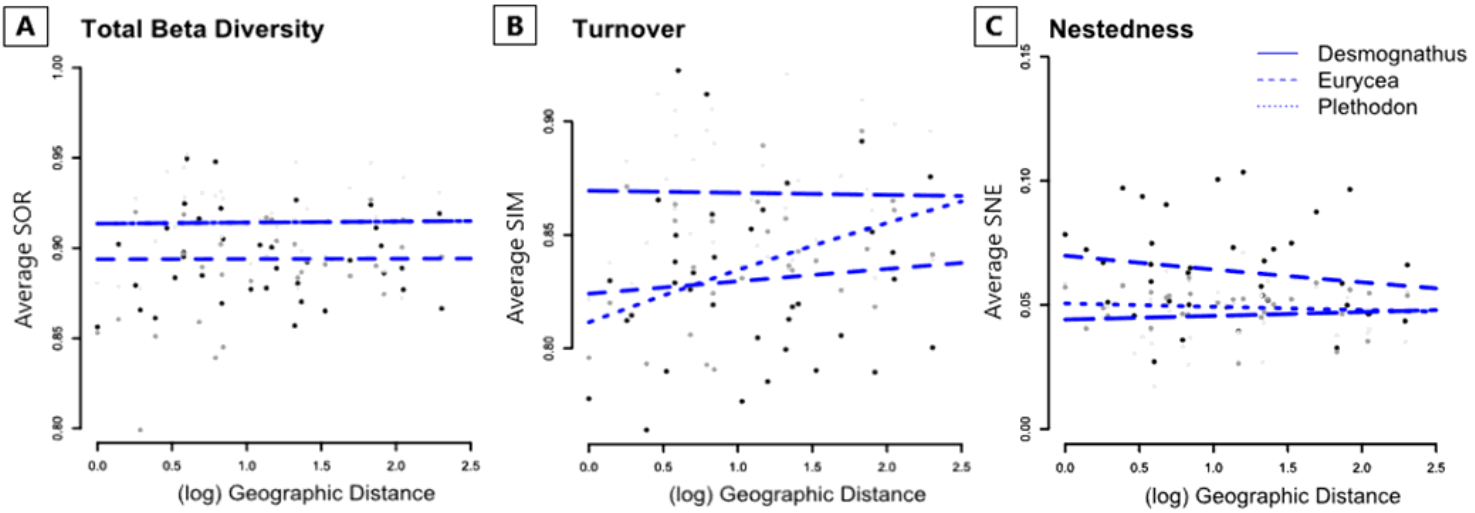

Figure 3

Generalized linear models of distance decay in (A) total β-diversity, (B) turnover, and (C) nestedness for skin-bacterial communities belonging to the salamander genera *Desmognathus*, *Eurycea*, and *Plethodon*

Supplementary Files

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- AppendixA.pdf