Diffuse interactions between symbiotic bacteria and salamanders

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Abstract

A growing body of evidence indicates that all animals and plants have intimate associations (symbioses) with microbes. Scientific opinion has shifted from viewing microbes primarily as pathogens to the idea that healthy animals and plants carry specialized communities of coevolving microorganisms. However, the generality of this proposition is unknown because surveys rarely compare host-associated microbial assemblages with those of similar host species or free-living assemblages in relevant microhabitats. To evaluate the specificity and consistency of microbial associations in salamanders, I analyzed just over 3.6 million 16S ribosomal RNA gene sequences from paired samples from host-associated bacteria on the skin of 62 salamanders and free-living bacteria from surfaces in their immediate home environments in the Great Smoky Mountains. I tested for community-level differences in relative abundance of bacterial taxa and population-level differences in haplotype frequencies within bacterial taxa. Comparing host-associated and free-living samples, I found evidence of bacterial specialization at both community and population levels, consistent with the idea that salamander skin is a selective medium supporting a distinctive microbial assemblage. However, I found no strong evidence of bacterial specialization among salamander species. Community differences among hosts were associated with spatial distance rather than host species or environmental similarity. Population-level differentiation among hosts was not apparent, consistent with frequent horizontal transmission. Salamander-bacterial associations are generally diffuse rather than specialized, with many host and symbiont taxa interacting in the same region.

Table of Contents

List of Figures

List of Tables

Introduction

Microbial symbiosis is a widespread, if not universal, phenomenon among multicellular organisms (McFall-Ngai 2002, Douglas 2008, Walter et al. 2011). This suggests that host-symbiont interactions are among the most important drivers of ecology and evolution on Earth. Symbiosis encompasses any intimate association between different kinds of organisms from parasitism to mutualism (Cariusab et al. 2001, Walter et al. 2011). Historically, microbiology has focused on parasite and pathogen interactions, but recent medical, ecological, and evolutionary research emphasizes the importance of mutualisms between bacteria and eukaryotes (Dethlefsen et al. 2007, Fraune and Bosch 2010). A more prevalent view is that healthy plants and animals carry intimate, specialized communities of coevolving microorganisms (Xu and Gordon 2003, Rosenberg et al. 2007, Turnbaugh et al. 2007, Gilbert et al. 2010) and might even be reconceived as communal metaorganisms (Dupré and O'Malley 2007, Rosenberg et al. 2007, Doolittle and Zhaxybayeva 2010). At the same time, traditional evolutionary ecology has embraced concepts of diffuse coevolution and interaction networks in which most interactions are generalized and geographically variable; specialization appears to be rare in eukaryotic interaction networks (Strauss and Irwin 2004, Thompson 2005a). Aside from a few well-studied pairwise interactions (Sachs et al. 2011), almost nothing is known about the consistency and specificity of microbial symbioses, making it difficult to determine whether the current difference in emphasis reflects real biological differences between macrobiological interaction networks and host-microbe relationships.

The consistency and specificity of an association are critical factors in the evolution of virulence or cooperation (Sachs et al. 2004), and although generalization can make interaction networks robust to perturbations, diffuse interactions provide many more opportunities for

conflict and cheating (Douglas 2008). Among the most widespread and important symbioses, examples of highly specialized partnerships (such as the *Rhizobium*-legume symbiosis and plant-chloroplast endosymbiosis) and diffuse networks (such as mycorrhizae (Selosse et al. 2006)) can be found. Although microbial symbionts of terrestrial animals have been widely documented (Harris et al. 2006, Lauer et al. 2007, Lauer et al. 2008, Costello et al. 2010, Walter et al. 2011), few studies have attempted to assess the extent of specialization vs. generalization at a community scale. The purpose of this study is to examine the consistency and specificity of host-microbe associations in a guild of related hosts (salamanders of the Southern Appalachians).

Amphibian skin appears to be a selective medium allowing and perhaps promoting growth of certain bacteria (Lauer et al. 2007, Lauer et al. 2008). Their skin is permeable for gas exchange and osmoregulation and physiologically is very active. Amphibians maintain a mucous layer that helps resist desiccation and often contains chemicals that are distasteful or toxic to predators (Petranka 1998, Dodd 2004). There is some evidence that bacteria in the mucous layer may help prevent fungal infections (Becker and Harris. 2010), and some researchers are working to develop probiotic treatments against the widespread chytrid *Batrachochytrium dendrobatidis*, a fungus implicated in amphibian decline and extinction (Harris et al. 2006). Thus, understanding the evolution and ecology of amphibian skin communities has both basic and applied significance. Highly specialized and distinctive host-associated communities would lend credence to the meta-organismal perspective and cast doubt on the hope for general-purpose probiotic management strategies. On the other hand, if the bacteria of amphibian skin tend to be generalists, interacting with multiple host genotypes and species in the context of a broader community, a generalist probiotic might indeed be a viable approach to *B. dendrobatidis* treatment.

Salamanders in the clade Plethodontidae are highly abundant and diverse throughout the southern Appalachian Mountains (Dodd 2004). Local communities often include several species of *Plethodon* and *Desmognathus* (Hairston 1987, Davic and Welsh 2004), in the Great Smoky Mountains. The common *Plethodon, P. jordani* and *P. teyahalee*, are known for their noxious skin secretions, presumed to function as deterrents to vertebrate predators (Petranka 1998, Dodd 2004) but also possibly having antimicrobial properties (Becker and Harris. 2010). *Desmognathus,* on the other hand, are palatable to potential predators (Brodie and Howard 1973, Petranka 1998). The two groups also differ in life history. Most *Desmognathus* have an aquatic larval stage, whereas *Plethodon* are direct-developers (Petranka 1998). Nevertheless, several *Desmognathus*, such as *D. ocoee* and *D. imitator*, spend substantial fractions of their lives in the same forest floor leaf litter habitats inhabited by *Plethodon* species.

Currently, it is unknown whether important symbionts and pathogens are specialized on particular salamander genotypes or whether they are broadly exchangeable. Lauer et al. (2007, 2008) noted similarities between samples of bacteria cultured from the skin of *Plethodon cinereus* and *Hemidactylium scutatum* from different localities in Virginia. However, standard culture conditions might tend to select a similar, non-random fraction from natural communities (Staley and Konopka 1985). McKenzie et al. (2011) compared skin samples from larvae of two pond-breeding frogs (*Lithobates pipiens* and *Pseudacris triseriata*) and one salamander species (*Ambystoma tigrinum*) using a culture-free assay (454 pyrosequencing of 16S ribosomal RNA gene from direct PCR of skin swabs). They found consistent differences between the frog and salamander larvae, but also noted that the same phylotype (identified as *Curvibacter*) was numerically dominant across all three species. Although these studies took steps to ensure sampling of resident bacteria rather than transient environmental contaminants, they did not

explicitly compare host-associated and free-living communities. Further, they did not directly test for signatures of specialization vs. generalization.

Here I undertook an extensive assessment of specialization and generalization of salamander skin bacteria at community and population levels. If salamanders generally harbor a highly specialized assemblage of bacteria, I expected to find large differences between salamander-associated and free-living samples, and between samples from different salamander species. Alternatively, if salamander-bacterial associations resemble most plant-animal interaction networks, different salamander species will tend to harbor the same kinds of bacteria. At the extreme, salamander-associated bacteria might be simple random samples from a local pool of free-living bacteria. I collected 62 paired samples (swabs of a salamander's skin and the cover object where it was found) from 25 sites nested within four localities in the Great Smoky Mountains National Park. I used high-throughput Illumina (Hi-seq) sequencing of the 16S ribosomal RNA gene as a culture-free assay of the bacteria in each sample. At the community level, I asked whether the relative abundance of bacterial OTUs (operational taxonomic units) differed between host-associated and free-living community samples, between sample localities, and between host species. I used indicator species analysis to screen for bacterial OTUs with strong affinities for salamanders in general or for particular species. At the population level, I tested whether host-associated populations of particular OTUs were genetically differentiated or homogeneous among hosts. These analyses provide an unprecedented assessment of where salamanders and their symbionts fall on the continuum between highly co-adapted insular metaorganisms and members of large diffuse interaction networks.

Methods

Permits

All research was conducted under North Carolina Wildlife Resources Commission Collection License Permit 11-SC00542, United States Department of the Interior National Park Service Scientific Research and Collecting Permit GRSM-2011-SCI-0062, and University of Tennessee Animal Care and Use Committee Protocol 2017-0611.

Study species

Salamanders of genera *Plethodon* and *Desmognathus* (family Plethodontidae) are medium sized, terrestrial salamanders (Dodd 2004). *Desmognathus ocoee* is located along the highest elevations in the Southern Appalachians and can be identified by their straightedge dorsal stripe that runs along the length of the body. *Plethodon teyahalee* is a medium to large sized slimy salamander with a deep blue to black body with lateral and dorsal white spots along the length of the body. *Plethodon jordani* is characterized by its blue to black body with bright red check patches (Dodd 2004, Chatfield et al. 2010). *Plethodon teyahalee, P. jordani,* and *Desmognathus ocoee* are generally abundant within the Great Smoky Mountains National Park and can be found under bark, logs, rocks, and other ground litter (Dodd 2004).

Sampling and sample sites

Salamander-cover object paired samples were collected from 25 collection sites nested in four localities in the Great Smoky Mountains National Park, North Carolina, during July 2011. The twenty-four sampling areas were located as eight evenly spaced sites along each of three 3.2 km transects: Noland Divide (5149-5907 ft), Kephart Prong (2783-3547 ft), and Mt. Sterling

Ridge (5204 – 5820 ft). Two salamanders were captured per collection site. Twelve salamander-cover object paired samples were also collected from Mt. Sterling Gap (elevation 3886 ft). Occasionally, two salamanders were captured under the same cover object. The number of total samples was 118, including 62 salamanders, 49 logs, and 7 rocks (Appendix B).

Microbial community collections

Salamanders were captured by hand and identified using morphological markers (Dodd 2004, Chatfield et al. 2010). Each salamander was rinsed with 15 mL of sterile, dechlorinated water to remove transient bacteria and swabbed on their ventral and lateral sides with two sterile cotton swabs following the protocol adapted from Lauer et al. (2007) for the collection of bacterial micro flora. The cover object, the bottom face of either a rock or log where the salamander was captured, was also swabbed with two sterile cotton swabs for the collection of environmental microbial communities. Salamanders were then released in the site of capture. All swabs were frozen at -20°C until DNA extraction.

Microbial community DNA was thawed and extracted from cotton swabs using the Qiagen QIAamp DNA Micro Kit (Germantown, MD, USA) according to the manufacturer's protocol for DNA extraction from wood-shaft cotton swabs.

Illumina sequencing preparation

DNA samples were prepared for Illumina sequencing following protocols adapted from Costello et al. (2010). PCR primers (F515/R806) were used to amplify the variable region V4 of the prokaryote 16S ribosomal RNA using standard conditions (Caporaso et al. 2011). To facilitate identification of samples after Illumina sequencing, 12 bp barcodes 0-117 (Caporaso et

al. 2011) were added to the 5' end of the F515 primer (Appendix C). A NanoDrop Micro-Volume UV-Vis Spectrophotometer was used to determine amplicon DNA concentration. All DNA amplicons were pooled in equalized DNA concentrations and cleaned using Qiagen QIAquick PCR Purification Kit (Germantown, MD, USA) according to the manufacture's protocols. These barcoded amplicons were pooled (after standardizing concentrations) and sent to the Yale Center for Genome Analysis (YCGA; West Haven, CT, USA) for paired-end sequencing on a Hi-Seq 2000. For all salamander and environment samples, PCR reactions were carried out in triplicate 24 μL reactions using 2.0 μL forward and reverse primers (10 μL final concentration), 1.0 μL template DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 μL GoTaq DNA Polymerase (5.0 μ / μL), 5.0 μL 5x GoTaq Reaction Buffer, and 10 μL DNA water. Thermo-cycling conditions included the following steps: 94°C for 3 min followed by 35 cycles of 94°C for 45 seconds, 50°C for 30 seconds, 72°C for 1 minute and 30 seconds, and a final elongation at 72°C for 10 min then held at 4°C. Presence of amplicons was checked by electrophoresis in 1.5% agarose and ethidium bromide staining for successful amplification. Triplicate PCR reactions were then pooled for each sample.

Sequence processing

Sequences were processed and run through quality control using the QIIME software package (Caporaso et al. 2011). Sequences were truncated after two consecutive low-quality base calls and removed from analysis if they contained more than one ambiguous base call. After parsing both forward and reverse reads by barcode, barcode and primer sequences were removed from each read. Reverse reads were reverse-complimented and concatenated with the corresponding forward read with the insertion of a gap to mark the joint. Forward and reverse

reads never overlapped and the unread gap between concatenated sequences ranged from approximately 124 to 200 bp. Concatenated reads of over 75 bp were clustered into OTUs using the UCLUST algorithm (Edgar 2010) and the Greengenes (DeSantis et al. 2006) reference set, trimmed to include the positions within the PCR product (Werner et al. 2012). For the main data set presented here, reads were counted only if they were within 94% identity of a sequence in the reference set and taxonomically identified according to the Greengenes taxonomy (Caporaso et al. 2010a). This procedure eliminates PCR chimeras between OTUs. I discarded very rare OTUs (less than 0.05% of the total abundance) because they have a high probability of being artifacts of sequencing errors (Caporaso et al. 2011, Werner et al. 2012). This resulted in a final data set of 3,645,376 reads assigned to 363 bacterial OTUs. I repeated the analysis with clustering at 97% to yield 777,278 reads assigned to 134 OTUs. These datasets were organized as community data matrices, containing counts of each OTU in each of the 118 samples.

Statistical analyses

Community-level analyses included pairwise comparisons of salamander samples with their corresponding cover object samples, ordination of all samples, regression of community dissimilarity against various predictors, and indicator species analysis to identify host-associated bacteria. Population-level analyses tested for genetic differentiation between host-associated vs. free-living samples and between host-species for particular bacterial OTUs.

The paired sampling scheme employed here provides a direct and powerful way to determine whether samples of bacteria from salamander skin are different from samples from their immediate environment. For each salamander-cover object pair, I performed a randomization test (Solow 1993, Manly 1997) with 10,000 permutations to obtain a *p*-value for

the null hypothesis that the two sets of bacterial sequence reads were sampled from the same distribution, accounting for differences in sample size (i.e., assuming any differences in the total number of reads were due to sampling, not biology). In practice, this was done as an RxC randomization (function chisq.test in R of 2 x *S* contingency tables (Hope 1968), where *S* is the number of OTUs in at least one of the samples. With 62 tests, a sequential Bonferroni correction (or other method for controlling false positive rates) should be used. In practice, all *p*-values were less than the Bonferonni critical value 0.05/62 = 0.00081.

As a global evaluation of whether there were consistent differences between salamander-associated and free-living samples, we performed unconstrained and constrained ordination (Anderson and Willis 2003), discriminant analysis (Borcard et al. 2011), and permutational MANOVA (Anderson 2001) using the R package *vegan* (Oksanen et al. 2011). For parametric methods (PCA and LDA), transformations were used to satisfy the assumptions of multivariate normality and homogeneity of variances (Legendre and Gallagher 2001). In practice, results were similar for all of the ecologically meaningful transformations suggested by Legendre and Gallagher (2001), and those based on Hellinger transformed $\log_{10}$ relative abundances are presented. Other analyses were based on pairwise community dissimilarity, calculated as the Horn-Morisita index (Jost et al 2011), Jaccard and Bray-Curtis distances (Borcard et al. 2011), and phylogenetic dissimilarities from UNIFRAC (Hamady et al. 2010) and PHYLOCOMM (Webb et al. 2008) applied to 100 rarefied datasets (Caporaso et al 2011). In practice, the choice of distance metric did not affect the outcome of the analyses. Distance-based RDA and perMANOVA both use permutations to test whether a significant portion of pairwise community dissimilarity could be explained by sample type (log, rock, or salamander) conditioned on transect, or by salamander species conditioned on transect. To ensure reasonable replication of

salamander species, these analyses were restricted to the three most commonly sampled species (*P. jordani*, *P. teyahalee*, and *D. ocoee*).

To evaluate whether similarity of salamander-associated bacterial communities was associated with spatial, environmental, and phylogenetic similarity, we used multiple regression on matrices (MRM) (Smouse et al. 1986, Legendre et al. 1994, Goslee and Urban 2007). The response variable was the matrix of pairwise overlap (Jost et al. 2011) between salamander associated bacterial samples. MRM results were compared for models that contained up to five explanatory variables: geographic distance between salamanders (km), pairwise community overlap between samples from their corresponding cover objects (perhaps the most relevant indicator of ecological similarity), transect (same or different), clade (originally scored as 0 for conspecifics, 1 for congeners, and 2 for different genera), and elevation difference (m). All variables were scaled to unit variance to provide standardized coefficients, and 1000 permutations were used to assess statistical significance. To evaluate the influence of multiple non-significant predictors on inference, I dropped the predictor with the highest *p*-value in a backwards stepwise fashion. These models were compared using AIC_c (computed as $n\log(\text{residual variance})+2kn/(n-k-1)$, where n is the true sample size of 62, and k is the number of estimated coefficients in the regression model (Burnham and Anderson 2004).

To identify bacterial OTUs with significant preference for salamanders vs. cover objects, indicator species analysis (De Cáceres and Legendre 2009, De Cáceres et al. 2010) was used with habitat types categorized as logs, rocks, *D. ocoee*, *P. jordani*, or *P. teyahalee*. Relative abundances were used to estimate group-equalized correlation coefficients (r_{pb}^{g}) for each category and all possible combinations, and permutation tests used to assess the statistical significance of the highest correlation for each OTU using the R package *indicspecies* (De

Cáceres and Legendre 2009). Our primary interest was in OTUs significantly associated with salamanders (group *D. ocoee* + *P. jordani*+ *P. teyahalee*).

To assess the possibility of specialization and local adaptation at a finer scale, population level tests for genetic differentiation were performed for four groups of OTUs: (i) the five OTUs with highest relative abundance on salamanders, (ii) the five OTUs with greatest association with salamanders according to indicator species analysis, (iii) the two OTUs clustering with *Janthinobacterium*, a candidate defensive mutualist (Lauer et al. 2008)), and (iv) five OTUs sampled at random from the remaining 351 OTUs. 16S ribosomal RNA gene sequence reads assigned to each of these 17 OTUs (at 94% similarity) were then separately clustered into *de novo* within-OTU clusters with 97% similarity using the UCLUST algorithm. I treated these clusters as alleles or "strains" within the OTUs defined by 94% similarity. Finer clustering (99% similarity or using raw reads) was not used to minimize any artificial diversity generated by sequencing error. This resulted in 17 datasets of within-OTU strain composition.

To test for genetic differentiation between host-associated and free-living populations, each within-OTU dataset was subjected to pairwise randomization tests (as above) for each salamander-cover object pair where the OTU was found. Results of these tests were summarized as Stouffer's combined *p*-value across pairs for each OTU (Whitlock 2005).

To test for genetic differentiation among populations associated with different salamander species, multiple regression was used on matrices to control for confounding effects of transect and spatial distance. Pairwise "strain" dissimilarity matrices were computed for each OTU (for each pair of salamanders on which the OTU was found) and regressed on geographic distance between salamanders (km), "transect distance" (0 for same, 1 for different), and "species distance" (0 for same, 1 for different).

To visualize patterns of association between bacterial haplotypes and the three focal salamander species, haplotype trees were estimated using raxml (Caporaso et al. 2010b) and compared against the three-taxon salamander tree by drawing tanglegrams (Jungles 1998) with lines from each bacterial haplotype to each salamander on which it was found.

Results and Discussion

Bacterial community similarity and differentiation

Overall, relative abundances of bacterial OTUs were strikingly similar between salamanders and cover objects (Fig. 1**)**. No taxa were restricted to, or excluded from, salamander skin, and the most abundant microbes tended to be most abundant in all habitats (Fig. 1). These similarities probably reflect both geographic association and habitat similarity between the moist surface of a salamander and the moist surface of a log or rock on the forest floor. However, relative abundances were not identical and significant statistical differences were detected between salamander-associated and cover object-associated samples in all 62 pairwise comparisons (randomization test $P < 0.0001$) and in global constrained ordination and perMANOVA analyses (Figs. 1-2).

Community similarity among samples was associated with locality, but not salamander species. Of the three principal salamander species in the study, *P. jordani* and *D. ocoee* were consistently found together at three of the sample localities and had very similar bacterial communities. *Plethodon teyahalee* was found only at Mt. Sterling Gap (where the other two were absent) and tended to have a more distinct bacterial community structure (Fig. 1), but no more so than expected for conspecifics in different localities (Fig. 2). Including salamander species as a factor did not improve the ordination or MANOVA models (Appendix D). This result is somewhat surprising given that both *Plethodon* are distasteful to vertebrate predators while *Desmognathus* is palatable (Brodie and Howard 1973, Petranka 1998, Dodd 2004). Whatever the differences in skin chemistry among these salamanders, they do not seem to affect bacterial community composition, though more subtle effects on local adaptation are possible.

Spatial patterns of community similarity

A multiple regression on matrices (MRM) was conducted to evaluate environmental, spatial, and phylogenetic predictors of symbiotic community similarity (Smouse et al. 1986, Legendre et al. 1994, Goslee and Urban 2007). The response variable was the matrix of pairwise overlap (Jost et al. 2011) between salamander associated bacterial samples. MRM results were compared for models that contained up to five explanatory variables: geographic distance between salamanders (km), pairwise community overlap between samples from their corresponding cover objects, transect, clade, and elevation (m). The only significant predictor of symbiotic community similarity was geographic distance (Table 1). Regardless of host species, salamander-associated communities from nearby individuals were more similar than communities from more distant individuals. That is, a *P. jordani* bacterial sample was often more similar to that of a neighboring *D. ocoee* than with another *P. jordani* from another area (Figs. 2-3). This spatial similarity of host-associated communities is not explained by spatial similarity of free living communities on cover objects (Table 1). These results suggest that direct or indirect interactions between neighboring salamanders have a significant homogenizing effect on their bacterial communities.

Indicator species

Although overall community structures are highly consistent among sample types, they were not identical (Fig. 1) and some bacterial OTUs consistently differed in relative abundance on salamanders vs. cover objects. Fifty bacterial OTUs were significantly associated with salamanders according to the point-biserial correlation (De Cáceres and Legendre 2009). Twelve of these OTUs were significantly associated with *P. teyahalee* alone, however, this

species was sampled from only a single location (Mt. Sterling Gap), making it impossible to distinguish species from locality effects (Appendix D). Only one OTU was weakly associated with *D. ocoee* alone, and none were significantly associated with *P. jordani* alone. The remaining 37 OTUs were associated with all three species as a group. The strongest associations had point-biserial correlations just over 0.7 for salamanders vs. cover objects (Table 2). These analyses provide no evidence of any bacterial OTU with consistently high abundance on only one salamander species. If bacteria with high host specificity exist in this system, they must be at very low abundance and/or patchy in terms of presence-absence on hosts.

Population level specificity and differentiation

To evaluate host-associated genetic differentiation within bacterial taxa, I focused on a subset of the 363 OTUs in the community dataset. These included the five most abundant OTUs on salamanders, the five most strongly associated with salamanders based on indicator species analysis, the two classified with *Janthinobacterium* (a group including candidate probiotics (Lauer et al. 2007, Lauer et al. 2008, Harris et al. 2009, Becker and Harris. 2010), and five random OTUs for comparison (Table 2). Fifteen of these 17 OTUs showed significant genetic differentiation between salamanders and cover objects (based on pairwise randomization tests). For example, even though *Nevskia ramosa* was the most commonly identified OTU on any substrate, there were consistent allele frequency differences between the *N. ramosa* samples taken from salamanders as opposed to those from logs or rocks. The community level differentiation between host-associated and free-living bacteria seems to be reflected within bacterial OTUs at the level of genotypes or "strains".

On the other hand, there was no strong evidence of genetic differentiation of bacteria among salamander host species (Table 2). Once spatial factors (distance or locality) were accounted for, host-associated bacteria on different host species were indistinguishable based on Bonferroni corrected tests. If one ignores the multiple testing problem only two OTUs showed any association between genetic distance and host species identity. Both of these OTUs were from among the five random OTUs, and one was positive and the other negative.

The many-to-many relationship between bacterial lineages and salamander species is illustrated by detailed mapping of individual interactions (Fig. 4). OTU 247980 (an unnamed clade in Sinobacteraceae) is a good example because it was consistently associated with salamanders but not so abundant as to make a graph too crowded (Table 2). Each genetic variant (97% similarity clusters within the set assigned to 247980) was found on multiple individuals from at least two salamander species and all individual salamanders with more than 10 reads in the group had more than one variant.

Population level patterns in *Janthinobacterium*

Janthinobacterium was identified as a candidate for providing antifungal resistance based on cultured samples from *P. cinereus* and *H. scutatum* (Lauer et al. 2007, Lauer et al. 2008, Becker and Harris. 2010) and has been proposed as a probiotic treatment to defend frogs and salamanders against *B. dendrobatidis* (Harris et al. 2009). Two clades of *Janthinobacterium* were found frequently on salamanders and cover objects. One had a week positive association with salamanders (Table 2), and both were similar to the other OTUs in showing allele frequency differences between salamander-associated and free-living samples, but no evidence of specialization or local adaptation among salamander species (Table 2). If some strains of

Janthinobacterium are naturally defensive mutualists with salamanders, my data indicate that they are probably generalists, and the relationship is facultative. However, inferences about function must be made with caution because horizontal gene transfer and homologous recombination make taxonomic assignments based on marker genes imperfect indicators of the traits that might be expressed by these organisms.

Conclusions

Interactions between organisms, including co-evolutionary interactions between host and symbionts, influence biological community dynamics and evolution (Thompson 1994, Thompson 2005b). Whether host-symbiont systems are highly integrated and exclusive units of organization or parts of more diffuse interaction webs is a key question for understanding how they relate to broader patterns of evolution and ecology. Overall, my results reveal strong similarities between salamander-associated communities and free-living communities in the same microhabitat and that relative abundances of bacterial OTUs were highly correlated. Although salamander-associated and free-living communities were not identical, their strong similarities suggest that damp surfaces in the southern Appalachians are occupied by a fairly predictable assemblage of bacteria. Community differences among hosts were associated with spatial distance rather than host species or environmental similarity, and population-level differentiation among hosts was not apparent. Salamander-bacterial associations sampled here are diffuse. Many host and symbiont taxa interact in the same region suggesting salamander-bacteria relationships resemble plant animal interaction networks much more than the metaorganisms envisioned by some. While these conclusions apply only to the skin, the results illustrate that sampling a few individuals of the same host species is not adequate to understand the evolution and ecology of microbial symbioses. In addition, the results from this study support efforts to discover generalist probiotics to help treat and prevent potential pathogens, such as *B. dendrobatidis*, because the skin-associated bacteria of distinct salamander lineages are highly consistent. The direction and rate of evolutionary change in these systems may depend more on community context and diffuse interactions rather than highly specialized and exclusive interactions.

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Appendix A

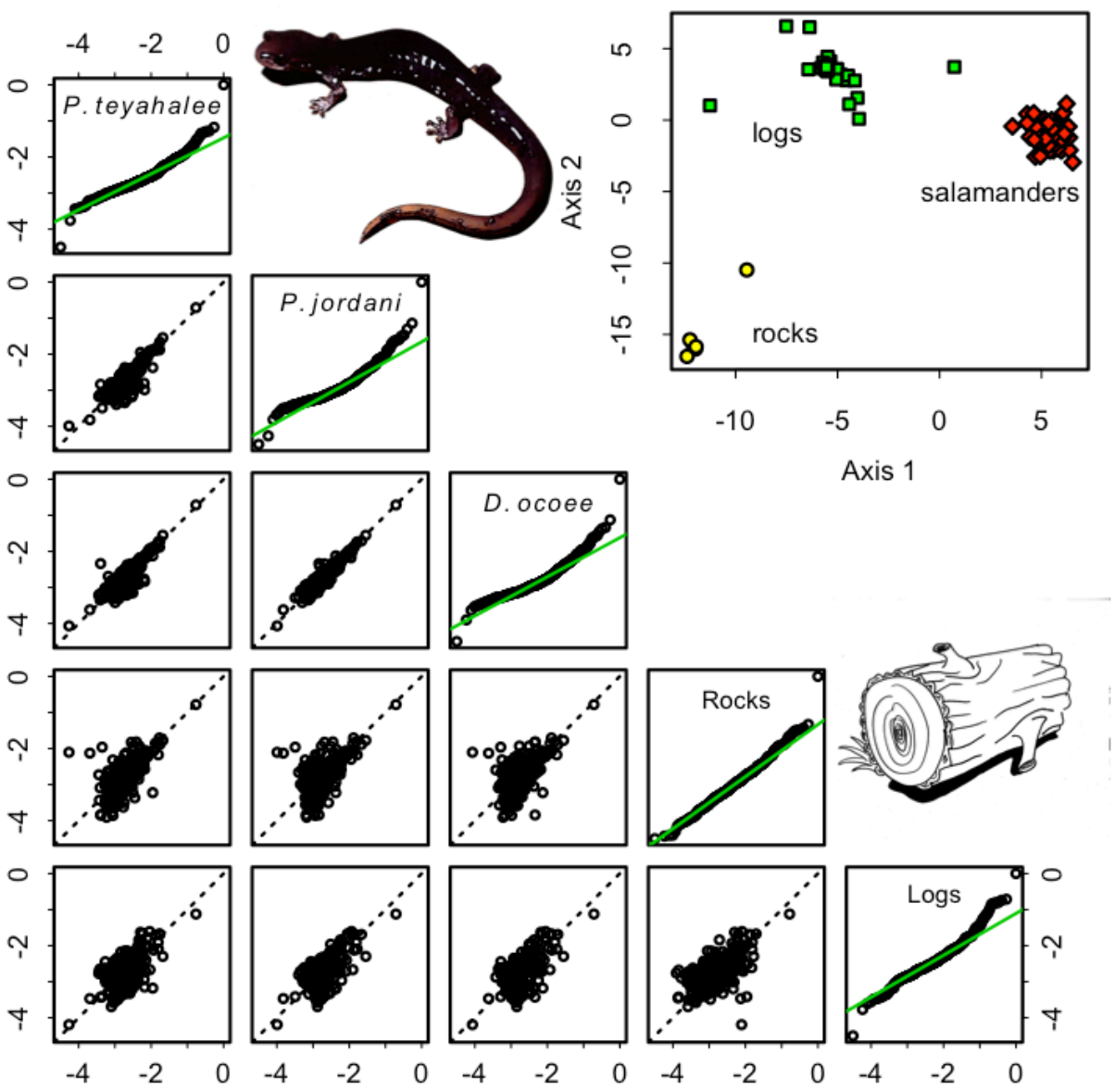

Figure 1. Bacterial community similarity and differentiation illustrated by correlated relative abundance distributions (scatter plot matrix) and constrained PCoA (inset). The diagonal panels are QQ-plots comparing the average relative abundance distribution for each sample type against a lognormal. Pairwise scatter plots of mean $\log_{10}$ relative abundance show a dashed line along the 1:1 expectation for identical communities. The inset (upper right) illustrates PCoA scores by sample from a constrained ordination of Morisita-Horn dissimilarities against sample type, conditioned on transect. Sample type was a significant explanatory factor (permutation test *P*-values <0.001 for permutational anova or PCoA).

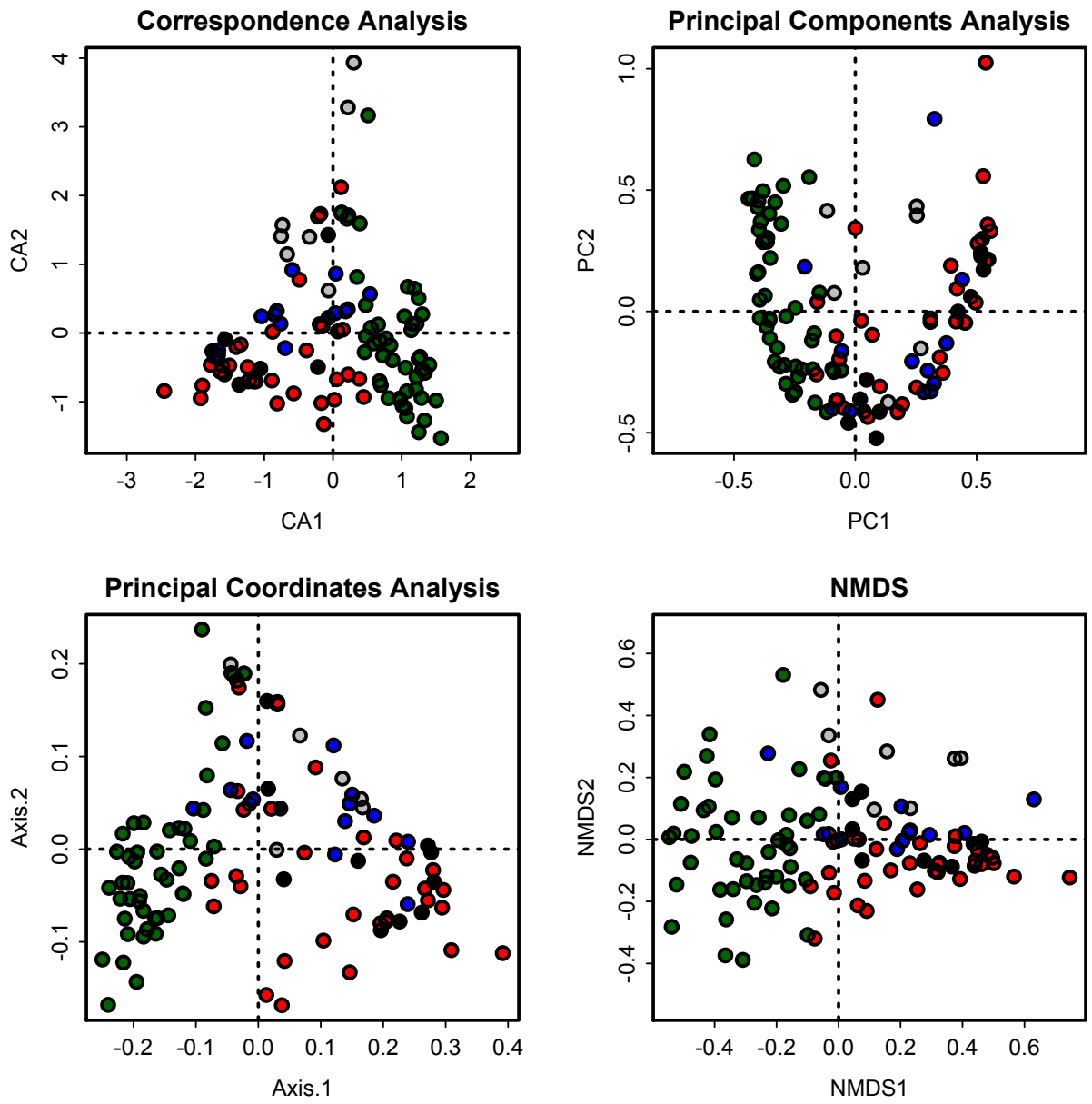

Figure 2. Diffuse symbiotic community representations among salamanders illustrated with unconstrained coordinate analysis.

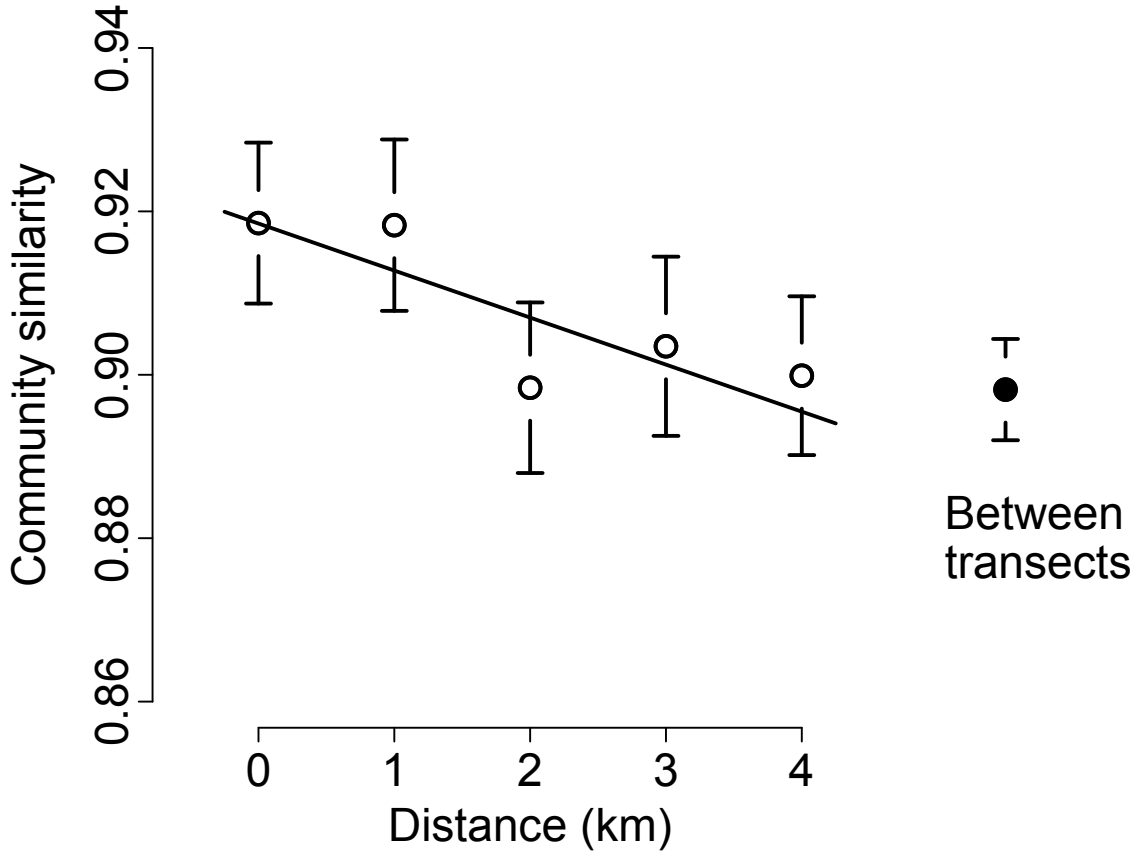

Figure 3. Symbiotic community similarity among salamanders declines over short geographic distance. Mean Morisita-Horn overlap and standard errors are shown. Other similarity metrics gave concordant results.

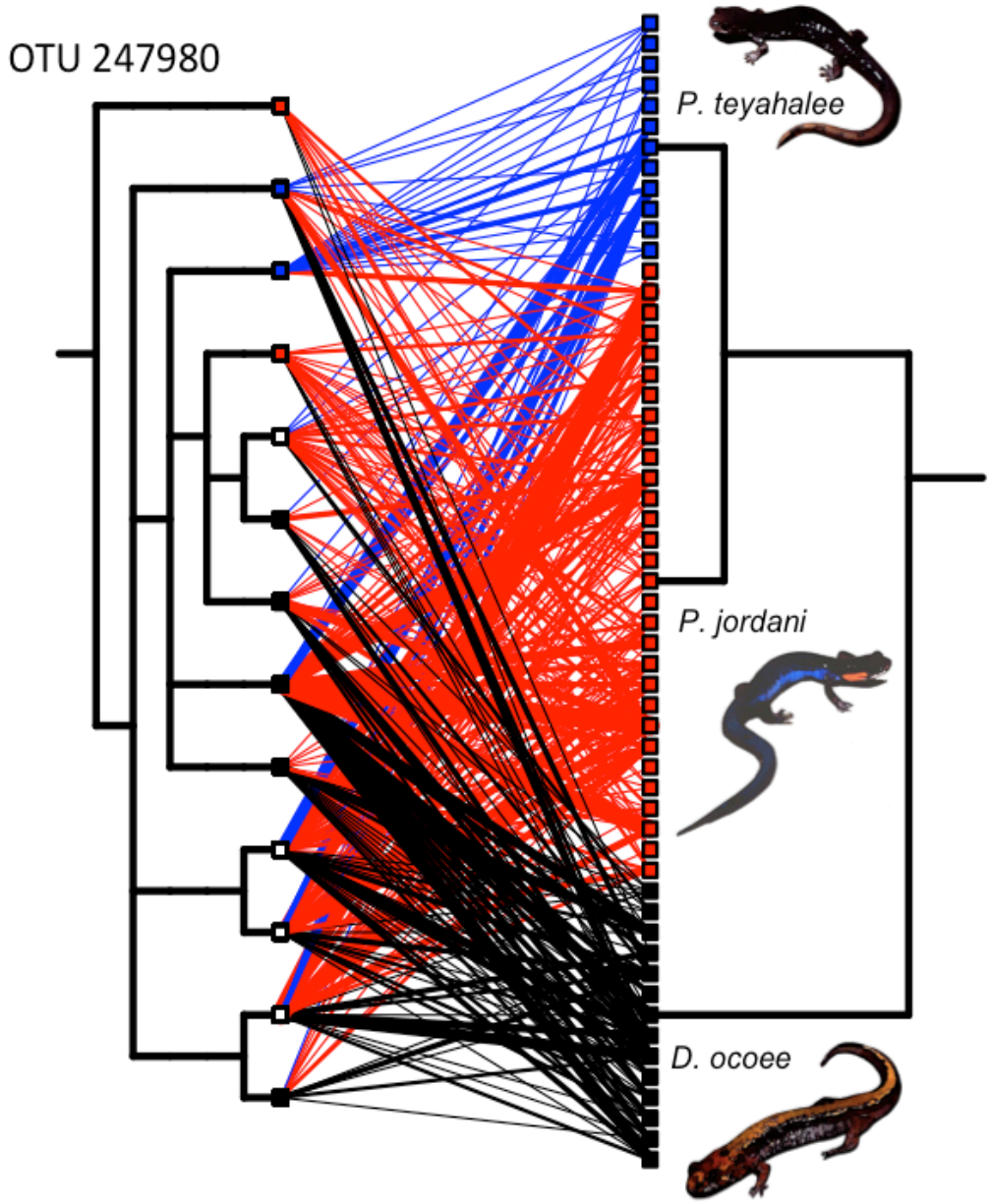

Figure 4. Diffuse relationships between salamanders and symbionts exemplified by many-to-many connections between three salamander species and 13 genetic variants of the bacterial OTU 247980 (an unnamed clade in Sinobacteraceae). Lines connecting bacterial genotypes to individual salamanders have width proportional to the $\log_e$ of their abundance on that individual. Line color corresponds to salamander species.

Table 1. Geographic distance is the only supported predictor of symbiotic community similarity

Model	ΔAIC_c	Rand. P-value	Coefficients: Intercept	Distance	Community	Transect	Clade	Elevation
5	8.061	0.0207	0.000	-0.080	0.119	-0.071	0.066	0.016
4	5.614	0.0113	0.000	**-0.089**	0.117	-0.056	0.067	
3	3.524	0.0128	0.000	**-0.093**	0.117	-0.044		
2.1	2.014	0.0006	0.000	**-0.091**		-0.055		
2.2	1.303	0.0141	0.000	**-0.123**	0.120			
1.1	0.047	0.0743	0.000		0.126			
1.2	0.000	0.0002	0.000	**-0.128**				

Multiple regression on matrices (MRM) results are compared for models including up to five explanatory variables. All variables were scaled to unit variance so that coefficients would be on the same scale. The response variable was the matrix of pairwise Horn overlap between salamander associated community samples. Distance is the geographic distance between salamanders. Community is the matrix of pairwise Horn overlap between community samples from each salamander's cover object. Transect is an indicator (0 same, 1 different). Clade is an ordinal phylogenetic distance between salamanders (0 same species, 1 between species within *Plethodon*, 2 between *Plethodon* and *Desmognathus*). Elevation is the difference in elevation between samples.

Table 2. Analysis of genetic differentiation within select OTUs

	Indicator value						Genetic differentiation			
	% Prevalence		% abundance		Association		Salamander v. cover		Salamander species	
	sal	cov	sal	cov	Rho	P	R2	P.pair	B	P.mrm
5 most abundant on salamanders										
7772: γ-proteobacteria: Sinobacteraceae: *Nevskia ramosa*	100.00	100.00	18.95	8.68	0.464	--[a]	0.174	<0.0001	-0.0126	0.180
94102: γ-proteobacteria: Sinobacteraceae: *Hydrocarboniphaga effusa*	100.00	92.73	2.68	0.65	0.574	--[a]	0.107	<0.0001	-0.0056	0.622
277971: γ-proteobacteria: Sinobacteraceae: unnamed	100.00	100.00	2.02	1.95	0.212	--[b]	0.172	<0.0001	-0.0312	0.117[e]
280477: Acidobacteria: Koribacteraceae: *Candidatus Koribacter* sp.	98.21	85.45	1.68	0.79	0.672	0.001	0.092	<0.0001	0.0000	0.998[e]
33919: β-proteobacteria: Burkholderaceae: *Burkholderia* sp.	100.00	100.00	1.57	1.28	0.310	--[a]	0.131	<0.0001	0.0290	0.173
5 best indicator OTUs										
292646: γ-proteobacteria: Pseudomonadaceae: *Pseudomonas pachastrellae*	80.36	27.27	0.12	0.03	0.732	0.001	0.038	<0.0001	0.0304	0.205
247980: γ-proteobacteria: Sinobacteraceae: unnamed	98.21	76.36	0.50	0.22	0.731	0.001	0.060	<0.0001	0.0080	0.718
576918: γ-proteobacteria: Chromatiaceae: *Rheinheimera* sp.	82.14	30.91	0.14	0.03	0.711	0.001	0.036	0.2018	-0.0012	0.948
236202: γ-proteobacteria: Moraxellaceae: *Acinetobacter johnsonii*	75.00	23.64	0.12	0.02	0.704	0.001	0.069	0.0003	-0.0293	0.193
400315: γ-proteobacteria: Pseudomonadaceae: *Pseudomonas* sp.	87.50	52.73	0.18	0.08	0.703	0.001	0.138	<0.0001	0.0064	0.667[e]
Janthinobacterium (β-proteobacteria: Oxalobacteraceae)										
225949 (sister to *J. lividum* 559256)	87.50	63.64	0.21	0.15	0.341	0.044	0.079	<0.0001	-0.0326	0.327
278562 (sister to *Janthinobacterium* sp. Marseilles 235428)	85.71	78.18	0.21	0.20	-0.285	ns	0.063	<0.0001	-0.0177	0.419[e]
5 random OTUs										
18609: α-proteobacteria: Hyphomonadaceae: unnamed	66.07	70.91	0.08	0.11	-0.269	ns	0.018	0.9999	0.1013	0.008[e]
139162: β-proteobacteria: *incertae sedis*: *Thiomonas* sp.	55.36	63.64	0.06	0.15	-0.126	--[c]	0.055	<0.0001	-0.0129	0.702
141085: α-proteobacteria: Acetobacteracaea: unnamed	83.93	89.09	0.14	0.41	-0.356	--[c]	0.044	<0.0001	-0.0025	0.555
2948: Chlamydiae: Chlamydiacaea: *Chlamydophila pneumoniae*	87.50	49.09	0.65	0.07	0.289	ns	0.038	<0.0001	-0.0177	0.615
202648: Chloroplast (probably green alga)	80.36	70.91	0.14%	0.18%	-0.342	--[d]	0.027	<0.0001	-0.0995	0.023[e]

[a] significantly associated with salamanders and rocks [b] significantly associated with salamanders and logs [c] significantly associated with logs [d] significantly associated with rocks [e] significant association between genetic differentiation and distance or transect.

Appendix B

Metadata of area of collection (transect), sample ID of microbial community sample from salamanders (ID), salamander species (description), elevation, latitude, and longitude of collection site, sample ID of environmental microbial community (cover ID), and Cover object type as a rock or log (cover).

Transect	ID	Elevation (ft)	Latitude	Longitude	Cover ID	Cover	Description
Noland Divide	45	5907	35.56727	-83.48152	33	rock	Plethodon jordani
Noland Divide	46	5907	35.56727	-83.48152	32	rock	Plethodon jordani
Noland Divide	30	5964	35.56725	-83.4815	31	log	Plethodon jordani
Noland Divide	37	5964	35.56725	-83.4815	38	rock	Desmognathus ocoee
Noland Divide	39	5664	35.56418	-83.47432	29	log	Plethodon jordani
Noland Divide	28	5664	35.56418	-83.47432	40	log	Plethodon jordani
Noland Divide	34	5664	35.56418	-83.47432	40	log	Desmognathus ocoee
Noland Divide	41	5520	35.56317	-83.4699	42	log	Plethodon jordani
Noland Divide	25	5520	35.56418	-83.4699	43	log	Desmognathus ocoee
Noland Divide	48	5425	35.56225	-83.46607	26	log	Desmognathus ocoee
Noland Divide	27	5425	35.56225	-83.46607	26	log	Plethodon jordani
Noland Divide	59	5367	35.55912	-83.4634	44	log	Plethodon jordani
Noland Divide	47	5367	35.55912	-83.4634	35	log	Plethodon jordani
Noland Divide	54	5209	35.55633	-83.46233	58	log	Plethodon jordani
Noland Divide	60	5209	35.55633	-83.46233	69	log	Desmognathus ocoee
Noland Divide	68	5149	35.55313	-83.46422	67	log	Plethodon jordani
Noland Divide	70	5149	35.55313	-83.46422	71	log	Plethodon jordani
Kephart Prong	49	2783	35.58623	-83.35855	51	log	Plethodon serratus
Kephart Prong	55	2783	35.58623	-83.35855	50	log	Eurycea wilderae
Kephart Prong	57	2870	35.58973	-83.3622	62	log	Plethodon jordani
Kephart Prong	72	2870	35.58973	-83.3622	52	rock	Desmognathus santeetlah
Kephart Prong	61	2961	35.59287	-83.36353	64	log	Desmognathus ocoee
Kephart Prong	66	2961	35.59287	-83.36353	63	log	Plethodon jordani
Kephart Prong	53	3094	35.59627	-83.36457	65	log	Plethodon jordani
Kephart Prong	74	3094	35.59627	-83.36457	73	log	Eurycea wilderae

Kephart Prong	76	3194	35.59937	-83.36613	96	log	Plethodon jordani
Kephart Prong	88	3194	35.59937	-83.36613	77	log	Desmognathus ocoee
Kephart Prong	79	3276	35.60273	-83.36752	85	log	Desmognathus ocoee
Kephart Prong	84	3276	35.60273	-83.36752	91	rock	Plethodon jordani
Kephart Prong	87	3397	35.60625	-83.3674	78	log	Desmognathus quadramaculatus
Kephart Prong	89	3397	35.60625	-83.3674	90	log	Plethodon jordani
Kephart Prong	93	3547	35.6097	-83.36885	94	rock	Plethodon jordani
Kephart Prong	80	3547	35.6097	-83.36885	92	rock	Desmognathus quadramaculatus
Mt. Sterling Ridge	115	5820	35.70263	-83.12197	107	log	Plethodon jordani
Mt. Sterling Ridge	83	5820	35.70263	-83.12197	106	log	Plethodon jordani
Mt. Sterling Ridge	112	5670	35.69952	-83.1244	95	log	Desmognathus ocoee
Mt. Sterling Ridge	104	5670	35.69952	-83.1244	105	log	Plethodon jordani
Mt. Sterling Ridge	82	5373	35.69678	-83.12617	114	log	Desmognathus ocoee
Mt. Sterling Ridge	81	5373	35.69678	-83.12617	109	log	Plethodon jordani
Mt. Sterling Ridge	113	5395	35.69387	-83.12835	116	log	Plethodon jordani
Mt. Sterling Ridge	110	5395	35.69387	-83.12835	118	log	Desmognathus ocoee
Mt. Sterling Ridge	100	5278	35.69123	-83.13178	117	log	Desmognathus ocoee
Mt. Sterling Ridge	98	5278	35.69123	-83.13178	97	log	Plethodon jordani
Mt. Sterling Ridge	102	5404	35.68887	-83.13537	103	log	Plethodon jordani
Mt. Sterling Ridge	119	5404	35.68887	-83.13537	108	log	Desmognathus, imitator
Mt. Sterling Ridge	99	5250	35.68735	-83.13895	101	log	Desmognathus ocoee
Mt. Sterling Ridge	135	5250	35.68735	-83.13895	120	log	Plethodon jordani
Mt. Sterling Ridge	111	5250	35.68735	-83.13895	120	log	Desmognathus ocoee
Mt. Sterling Ridge	130	5204	35.68597	-83.14305	131	log	Plethodon jordani
Mt. Sterling Ridge	127	5204	35.68597	-83.14305	138	log	Plethodon jordani
Mt Sterling Gap	122	3886	35.70003	-83.0974	141	log	Plethodon teyahalee
Mt Sterling Gap	121	3886	35.70003	-83.0974	120	log	Plethodon teyahalee
Mt Sterling Gap	144	3886	35.70003	-83.0974	133	log	Plethodon teyahalee
Mt Sterling Gap	126	3886	35.70003	-83.0974	134	log	Plethodon teyahalee
Mt Sterling Gap	123	3886	35.70003	-83.0974	136	log	Plethodon teyahalee
Mt Sterling Gap	128	3886	35.70003	-83.0974	124	log	Plethodon teyahalee

Mt Sterling Gap	129	3886	35.70003	-83.0974	140	log	Plethodon teyahalee
Mt Sterling Gap	132	3886	35.70003	-83.0974	140	log	Plethodon teyahalee
Mt Sterling Gap	137	3886	35.70003	-83.0974	142	log	Plethodon teyahalee
Mt Sterling Gap	196	3886	35.70003	-83.0974	143	log	Plethodon teyahalee
Mt Sterling Gap	195	3886	35.70003	-83.0974	197	log	Plethodon teyahalee
Mt Sterling Gap	193	3886	35.70003	-83.0974	197	log	Plethodon teyahalee

Appendix C

Primers used for the paired-end 16S community sequencing on the Illumina platform using the bacterial/archaeal primer F515/R806.

Primer	Sequence
515F	GTGCCAGCMGCCGCGGTAA
806R	GGACTACHVGGGTWTCTAAT
515fbc0	TCCCTTGTCTCCGTGCCAGCMGCCGCGGTAA
515fbc1	ACGAGACTGATTGTGCCAGCMGCCGCGGTAA
515fbc2	GCTGTACGGATTGTGCCAGCMGCCGCGGTAA
515fbc3	ATCACCAGGTGTGTGCCAGCMGCCGCGGTAA
515fbc4	TGGTCAACGATAGTGCCAGCMGCCGCGGTAA
515fbc5	ATCGCACAGTAAGTGCCAGCMGCCGCGGTAA
515fbc6	GTCGTGTAGCCTGTGCCAGCMGCCGCGGTAA
515fbc7	AGCGGAGGTTAGGTGCCAGCMGCCGCGGTAA
515fbc8	ATCCTTTGGTTCGTGCCAGCMGCCGCGGTAA
515fbc9	TACAGCGCATACGTGCCAGCMGCCGCGGTAA
515fbc10	ACCGGTATGTACGTGCCAGCMGCCGCGGTAA
515fbc11	AATTGTGTCGGAGTGCCAGCMGCCGCGGTAA
515fbc12	TGCATACACTGGGTGCCAGCMGCCGCGGTAA
515fbc13	AGTCGAACGAGGGTGCCAGCMGCCGCGGTAA
515fbc14	ACCAGTGACTCAGTGCCAGCMGCCGCGGTAA
515fbc15	GAATACCAAGTCGTGCCAGCMGCCGCGGTAA
515fbc16	GTAGATCGTGTAGTGCCAGCMGCCGCGGTAA
515fbc17	TAACGTGTGTGCGTGCCAGCMGCCGCGGTAA
515fbc18	CATTATGGCGTGGTGCCAGCMGCCGCGGTAA
515fbc19	CCAATACGCCTGGTGCCAGCMGCCGCGGTAA
515fbc20	GATCTGCGATCCGTGCCAGCMGCCGCGGTAA
515fbc21	CAGCTCATCAGCGTGCCAGCMGCCGCGGTAA
515fbc22	CAAACAACAGCTGTGCCAGCMGCCGCGGTAA
515fbc23	GCAACACCATCCGTGCCAGCMGCCGCGGTAA
515fbc24	GCGATATATCGCGTGCCAGCMGCCGCGGTAA
515fbc25	CGAGCAATCCTAGTGCCAGCMGCCGCGGTAA
515fbc26	AGTCGTGCACATGTGCCAGCMGCCGCGGTAA
515fbc27	GTATCTGCGCGTGTGCCAGCMGCCGCGGTAA
515fbc28	CGAGGGAAAGTCGTGCCAGCMGCCGCGGTAA
515fbc29	CAAATTCGGGATGTGCCAGCMGCCGCGGTAA
515fbc30	AGATTGACCAACGTGCCAGCMGCCGCGGTAA
515fbc31	AGTTACGAGCTAGTGCCAGCMGCCGCGGTAA
515fbc32	GCATATGCACTGGTGCCAGCMGCCGCGGTAA
515fbc33	CAACTCCCGTGAGTGCCAGCMGCCGCGGTAA
515fbc34	TTGCGTTAGCAGGTGCCAGCMGCCGCGGTAA
515fbc35	TACGAGCCCTAAGTGCCAGCMGCCGCGGTAA
515fbc36	CACTACGCTAGAGTGCCAGCMGCCGCGGTAA
515fbc37	TGCAGTCCTCGAGTGCCAGCMGCCGCGGTAA
515fbc38	ACCATAGCTCCGGTGCCAGCMGCCGCGGTAA
515fbc39	TCGACATCTCTTGTGCCAGCMGCCGCGGTAA
515fbc40	GAACACTTTGGAGTGCCAGCMGCCGCGGTAA

515fbc41	GAGCCATCTGTAGTGCCAGCMGCCGCGGTAA
515fbc42	TTGGGTACACGTGTGCCAGCMGCCGCGGTAA
515fbc43	AAGGCGCTCCTTGTGCCAGCMGCCGCGGTAA
515fbc44	TAATACGGATCGGTGCCAGCMGCCGCGGTAA
515fbc45	TCGGAATTAGACGTGCCAGCMGCCGCGGTAA
515fbc46	TGTGAATTCGGAGTGCCAGCMGCCGCGGTAA
515fbc47	CATTCGTGGCGTGTGCCAGCMGCCGCGGTAA
515fbc48	TACTACGTGGCCGTGCCAGCMGCCGCGGTAA
515fbc49	GGCCAGTTCCTAGTGCCAGCMGCCGCGGTAA
515fbc50	GATGTTCGCTAGGTGCCAGCMGCCGCGGTAA
515fbc51	CTATCTCCTGTCGTGCCAGCMGCCGCGGTAA
515fbc52	ACTCACAGGAATGTGCCAGCMGCCGCGGTAA
515fbc53	ATGATGAGCCTCGTGCCAGCMGCCGCGGTAA
515fbc54	GTCGACAGAGGAGTGCCAGCMGCCGCGGTAA
515fbc55	TGTCGCAAATAGGTGCCAGCMGCCGCGGTAA
515fbc56	CATCCCTCTACTGTGCCAGCMGCCGCGGTAA
515fbc57	TATACCGCTGCGGTGCCAGCMGCCGCGGTAA
515fbc58	AGTTGAGGCATTGTGCCAGCMGCCGCGGTAA
515fbc59	ACAATAGACACCGTGCCAGCMGCCGCGGTAA
515fbc60	CGGTCAATTGACGTGCCAGCMGCCGCGGTAA
515fbc61	GTGGAGTCTCATGTGCCAGCMGCCGCGGTAA
515fbc62	GCTCGAAGATTCGTGCCAGCMGCCGCGGTAA
515fbc63	AGGCTTACGTGTGTGCCAGCMGCCGCGGTAA
515fbc64	TCTCTACCACTCGTGCCAGCMGCCGCGGTAA
515fbc65	ACTTCCAACTTCGTGCCAGCMGCCGCGGTAA
515fbc66	CTCACCTAGGAAGTGCCAGCMGCCGCGGTAA
515fbc67	GTGTTGTCGTGCGTGCCAGCMGCCGCGGTAA
515fbc68	CCACAGATCGATGTGCCAGCMGCCGCGGTAA
515fbc69	TATCGACACAAGGTGCCAGCMGCCGCGGTAA
515fbc70	GATTCCGGCTCAGTGCCAGCMGCCGCGGTAA
515fbc71	CGTAATTGCCGCGTGCCAGCMGCCGCGGTAA
515fbc72	GGTGACTAGTTCGTGCCAGCMGCCGCGGTAA
515fbc73	ATGGGTTCCGTCGTGCCAGCMGCCGCGGTAA
515fbc74	TAGGCATGCTTGGTGCCAGCMGCCGCGGTAA
515fbc75	AACTAGTTCAGGGTGCCAGCMGCCGCGGTAA
515fbc76	ATTCTGCCGAAGGTGCCAGCMGCCGCGGTAA
515fbc77	AGCATGTCCCGTGTGCCAGCMGCCGCGGTAA
515fbc78	GTACGATATGACGTGCCAGCMGCCGCGGTAA
515fbc79	GTGGTGGTTTCCGTGCCAGCMGCCGCGGTAA
515fbc80	TAGTATGCGCAAGTGCCAGCMGCCGCGGTAA
515fbc81	TGCGCTGAATGTGTGCCAGCMGCCGCGGTAA
515fbc82	ATGGCTGTCAGTGTGCCAGCMGCCGCGGTAA
515fbc83	GTTCTCTTCTCGGTGCCAGCMGCCGCGGTAA
515fbc84	CGTAAGATGCCTGTGCCAGCMGCCGCGGTAA
515fbc85	GCGTTCTAGCTGGTGCCAGCMGCCGCGGTAA
515fbc86	GTTGTTCTGGGAGTGCCAGCMGCCGCGGTAA
515fbc87	GGACTTCCAGCTGTGCCAGCMGCCGCGGTAA
515fbc88	CTCACAACCGTGGTGCCAGCMGCCGCGGTAA

515fbc89	CTGCTATTCCTCGTGCCAGCMGCCGCGGTAA
515fbc90	ATGTCACCGCTGGTGCCAGCMGCCGCGGTAA
515fbc91	TGTAACGCCGATGTGCCAGCMGCCGCGGTAA
515fbc92	AGCAGAACATCTGTGCCAGCMGCCGCGGTAA
515fbc93	TGGAGTAGGTGGGTGCCAGCMGCCGCGGTAA
515fbc94	TTGGCTCTATTCGTGCCAGCMGCCGCGGTAA
515fbc95	GATCCCACGTACGTGCCAGCMGCCGCGGTAA
515fbc96	TACCGCTTCTTCGTGCCAGCMGCCGCGGTAA
515fbc97	TGTGCGATAACAGTGCCAGCMGCCGCGGTAA
515fbc98	GATTATCGACGAGTGCCAGCMGCCGCGGTAA
515fbc99	GCCTAGCCCAATGTGCCAGCMGCCGCGGTAA
515fbc100	GATGTATGTGGTGTGCCAGCMGCCGCGGTAA
515fbc101	ACTCCTTGTGTTGTGCCAGCMGCCGCGGTAA
515fbc102	GTCACGGACATTGTGCCAGCMGCCGCGGTAA
515fbc103	GCGAGCGAAGTAGTGCCAGCMGCCGCGGTAA
515fbc104	ATCTACCGAAGCGTGCCAGCMGCCGCGGTAA
515fbc105	ACTTGGTGTAAGGTGCCAGCMGCCGCGGTAA
515fbc106	TCTTGGAGGTCAGTGCCAGCMGCCGCGGTAA
515fbc107	TCACCTCCTTGTGTGCCAGCMGCCGCGGTAA
515fbc108	GCACACCTGATAGTGCCAGCMGCCGCGGTAA
515fbc109	GCGACAATTACAGTGCCAGCMGCCGCGGTAA
515fbc110	TCATGCTCCATTGTGCCAGCMGCCGCGGTAA
515fbc111	AGCTGTCAAGCTGTGCCAGCMGCCGCGGTAA
515fbc112	GAGAGCAACAGAGTGCCAGCMGCCGCGGTAA
515fbc113	TACTCGGGAACTGTGCCAGCMGCCGCGGTAA
515fbc114	CGTGCTTAGGCTGTGCCAGCMGCCGCGGTAA
515fbc115	TACCGAAGGTATGTGCCAGCMGCCGCGGTAA
515fbc116	CACTCATCATTCGTGCCAGCMGCCGCGGTAA
515fbc117	GTATTTCGGACGGTGCCAGCMGCCGCGGTAA

Appendix D

Table D1. Distance-based redundancy analysis (DB-RDA) of the full dataset testing sample type (salamander, log, or rock) as a predictor of bacterial relative abundance, conditioned on sample locality. The p-value was obtained from 10,000 permutations. PermANOVA gave concordant results.

	DF	Sum of squares	*F*	r^2	p-value
Model	2	2.8177	61.716	0.61211	<0.0001
Residual	112	2.5567			

Table D2. Distance-based redundancy analysis (DB-RDA) of the full dataset testing locality as a predictor of bacterial relative abundance, conditioned on sample type. The p-value was obtained from 10,000 permutations. PermANOVA gave concordant results.

	DF	Sum of squares	*F*	r^2	p-value
Model	3	0.20959	3.0604	0.06283	0.0028
Residual	112	2.55674			

Table D3. Distance-based redundancy analysis (DB-RDA) testing salamander species as a predictor of bacterial relative abundance, conditioned on sample locality. The p-value was obtained from 10,000 permutations. PermANOVA gave concordant results. Only samples from *Plethodon jordani*, *P. teyahalee*, and *Desmognathus ocoee* were included.

	DF	Sum of squares	*F*	r^2	p-value
Model	1	0.00266	0.1168	0.02569	0.9335
Residual	51	1.16034			

Vita

Amanda Allison grew up in Myrtle Beach, SC. She completed the International Baccalaureate Diploma Program and graduated from Socastee High School in Myrtle Beach, SC in 2006. She graduated from Clemson University in 2010 with a Bachelors of Science degree in Genetics. In Fall 2010, she accepted a graduate teaching assistantship at the University of Tennessee, Knoxville in the department of Ecology and Evolutionary Biology. Amanda received her Masters of Science degree in Ecology and Evolutionary Biology in August 2012.