Intraspecific Variation in the Skin-Associated Microbiome of a Terrestrial Salamander

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Abstract Resident microbial communities living on amphibian skin can have significant effects on host health, yet the basic ecology of the host-microbiome relationship of many amphibian taxa is poorly understood. We characterized intraspecific variation in the skin microbiome of the salamander *Ensatina eschscholtzii xanthoptica*, a subspecies composed of four genetically distinct populations distributed throughout the San Francisco Bay Area and the Sierra Nevada mountains in California, USA. We found that salamanders from four geographically and genetically isolated populations harbor similar skin microbial communities, which are dominated by a common core set of bacterial taxa. Additionally, within a population, the skin microbiome does not appear to differ significantly between salamanders of different ages or sexes. In all cases, the salamander skin microbiomes were significantly different from those of the surrounding terrestrial environment. These results suggest that the relationship between *E. e. xanthoptica* salamanders and their resident skin microbiomes is conserved, possibly indicating a stable mutualism between the host and microbiome.

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Introduction

Communities of microbes living on and within other organisms play a significant role in many aspects of host life history, from development and physiology to health and behavior [1–3]. The composition of these resident microbial communities (termed microbiomes) is influenced by many factors, including the host immune system, the environment, and interactions with non-resident microbes [2, 4–6]. However, the drivers of species assemblage and maintenance of host-associated microbiomes in natural systems remain unclear [7, 8].

Amphibian skin has recently emerged as a unique model system in which to study host-associated microbiomes. As amphibians lack protective structures found in other vertebrates such as scales, fur, or feathers, the skin plays a disproportionately large role in physiological processes such as respiration and osmotic balance [7, 9]. Additionally, microbial communities living on amphibian skin have been shown to significantly affect health and disease resistance [10, 11]. As a result, these host microbial communities are an important target for conservation applications, such as efforts aimed at mitigation of a deadly skin disease [12–14]. However, before such conservation strategies can be effectively employed, it is necessary to understand the structure and dynamics of amphibian skin microbial communities in natural systems [15].

Findings from recent studies characterizing amphibian skin microbiomes in natural systems have not been entirely congruous. For example, some studies have shown that the skin microbiome assemblage is primarily determined by host species identity [7, 8, 16], while others show that the host's habitat is a primary determinant of the skin microbiome assemblage [5,

17]. Such seemingly conflicting evidence may be due to the fact that these studies have focused on different taxa, ranging from terrestrial forest salamanders to montane aquatic frogs. To tease apart the effects of host-associated and environmental factors on the skin microbiome, focusing on microbiome variation within a species may be an effective strategy.

Studies of intraspecific variation in microbiome composition provide a model that minimizes differences in physiology, behavior, and ecology which are present in any study of multiple host species. Comparisons across host populations that are similar in all regards except geographic distribution can test whether factors like habitat and environmental microbes influence the skin microbiome. Such an approach can thus provide insight into how conserved the host-microbiome relationship is across the host range.

In addition, intraspecific studies can allow researchers to explore variation in skin microbiome composition across host traits such as sex and age. While sex-specific microbiome variation has not yet been investigated in amphibians, such variation has been observed in the gut and skin of humans and mice [18–20]. It is possible that sex-based differences in hormone and pheromone production present in amphibians may impact the composition of the amphibian skin microbiome as well [21–23]. The effects of age on the amphibian microbiome have been more thoroughly studied, but research has primarily focused on species with aquatic larval stages. In several such species, results indicate a shift in skin microbiome composition between larval and adult life stages [7, 24–26]. To our knowledge, only one study has investigated a direct-developing amphibian (the frog *Eleutherodactylus coqui*), and it showed conflicting results: some analyses suggested that the microbiome differed between adults and juveniles, but other analyses showed no difference [27]. Thus, it remains unclear whether the microbiome shifts through growth and development in direct-developing species.

In this study, we investigated intraspecific variation in the skin microbiome of the yellow-eyed ensatina (*Ensatina eschscholtzii xanthoptica* Gray 1850), a terrestrial direct-developing plethodontid salamander. This subspecies is composed of four genetically and geographically distinct populations distributed in the San Francisco Bay Area and the Sierra Nevada mountains [28]. We used 16S amplicon sequencing to characterize and compare skin microbial communities between populations, age classes, and sexes to explore factors that may be driving skin microbiome assemblage in this salamander.

Our study goals were threefold. First, we tested whether individuals from different populations within this subspecies harbor microbiomes that are similar or distinct from one another. Second, we tested whether the microbiome varies significantly between demographic groups, specifically age classes (juvenile or adult) and sexes. Lastly, we compared skin microbiome composition to that of the surrounding environment (soil) to determine whether salamanders harbor a microbial community that is significantly different from that of their habitat.

Methods

Field Sampling

The subspecies *E. e. xanthoptica* is found throughout the San Francisco Bay Area and the Sierra Nevada mountains in California, USA. Individuals can be found under fallen logs and other debris in forested areas [29]. The subspecies is composed of four genetically and geographically distinct populations: North Bay, East Bay, South Bay, and Sierra Nevada (Fig. 1) [28]. For this study, we sampled 10–11 adult salamanders from each of the four populations. We sampled salamanders opportunistically, and distances between salamanders sampled varied. It is therefore possible that some individuals in a population are more closely related than others; however, we did not perform genetic analysis of the host salamanders, so we are unable to determine relatedness.

To compare differences between sexes and age classes, we expanded our sampling of the North Bay population to include equal numbers of males, females, and juveniles ($n = 10$, $n = 11$, $n = 10$, respectively). We chose to use the North Bay population for this comparison because it had the highest salamander abundance; we were unable to find sufficient numbers for age and sex comparisons in the other populations. Nonetheless, we have no reason to expect patterns to differ between populations.

To explore the effect of environmental microbes on skin microbiota, we also analyzed five soil samples from each population (20 soil samples total). A total of 64 salamander skin swabs and 20 soil samples were used in this study.

Due to drought conditions, populations were sampled at different times of the year as follows: East Bay, March 2014; South Bay, April–May and September 2014; North Bay, December 2014; and Sierra Nevada March 2015.

Salamanders were captured by hand using new nitrile gloves, rinsed with 18 MΩ/cm MilliQ water (25 ml for juveniles, 50 ml for adults), and swabbed using a sterile synthetic cotton swab, stroking 30 times (10 ventral, 10 dorsal, 5 each side). Soil samples were collected from the soil surface directly under the cover object (e.g., rock or log) of each salamander within approximately 15 cm of an individual's point of capture. Soil was collected in sterile 1.5 ml Eppendorf tubes. Approximately 0.25 g of each soil sample was used for DNA extraction. Animals were released within approximately 30 min of capture to their original cover objects. Swabs and soil samples were immediately placed on dry ice and then frozen at −80 °C until DNA extraction.

Fig. 1 Map of sampling locations. Each *point* represents an individual salamander sampled; many overlap due to close proximity (East Bay *n* = 11, North Bay *n* = 10, Sierra Nevada *n* = 11, South Bay *n* = 10). *Colors* correspond to populations
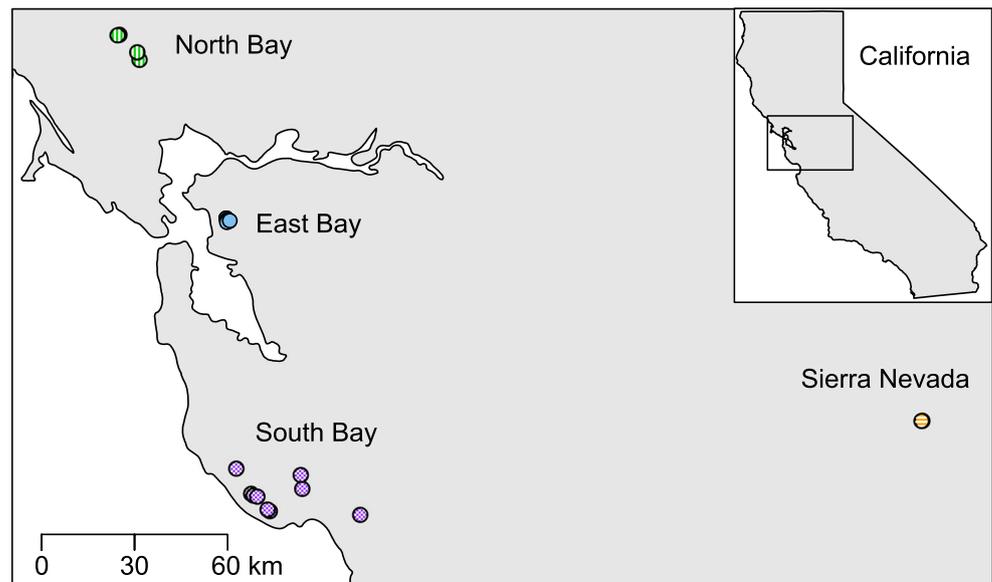
Microbiome DNA Extraction, Amplification, and Sequencing

Total genomic DNA from skin swabs and soil samples was extracted using the PowerSoil DNA Isolation Kit according to the manufacturer protocol (MoBio Laboratories, Carlsbad, CA, USA). Five soil samples were randomly chosen from each population for microbiome analysis. We PCR-amplified the V3–V4 region of the bacterial 16S rRNA gene using Illumina primers (see Supplementary Table 1) according to the recommended Illumina protocol, with a slight modification to maximize PCR product (specifically, we increased the number of cycles for Index PCR from 8 to 12) (Illumina Inc., San Diego, CA, USA). Samples were individually barcoded during the index PCR step using sample-specific pairs of dual-indexing primers from the Illumina Nextera Index Kit (see Supplementary Table 1). All samples were run in triplicate to limit PCR bias and produce sufficient quantities of PCR product. Amplified product was quantified using the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA). Samples were then pooled at equimolar concentrations and run on the Illumina MiSeq at the San Francisco State University Genomics and Transcriptomics Analysis Core. 16S amplicon libraries have inherently low diversity, so we added 25% PhiX to the solution before loading the MiSeq to ensure proper cluster density. We sequenced 300 bp paired-end reads, which were demultiplexed to produce final reads of ~460 bp for the region of interest.

Bioinformatics and Statistical Analysis

The bioinformatics pipeline QIIME [30] was used for all sequence analyses unless otherwise stated. Sequences were demultiplexed and quality filtered using default protocol (q20 threshold), and the resulting reads were clustered into operational taxonomic units (OTUs) based on 97% similarity, using the cluster seed as the reference sequence. OTUs were then assigned taxonomy using the Greengenes reference database (Greengenes Database Consortium, 2015). For sequences that did not match the reference database, OTUs were clustered de novo, and the centroid of the cluster was chosen as the representative sequence. Any OTUs that were not identified as bacterial were removed. OTUs present in only one sample overall or with less than 100 reads were excluded from our analysis (0.05% threshold) [31].

We used three separate approaches to address our three experimental goals. First, to compare microbiome variation between salamander populations, we analyzed alpha and beta diversity measures across our four populations (*n* = 42). For the North Bay, we randomly selected five male and five female adults for this analysis. Second, to test for microbiome differences between sexes and age groups, we analyzed microbial diversity within the North Bay population (*n* = 31). Third, to test whether the microbiome of salamanders is significantly distinct from that of their habitat (soil), we compared salamander samples from each population (*n* = 42) to five soil samples from each population (*n* = 20). We rarefied the number of reads per sample for each analysis separately to ensure equivalent sampling (populations = 31,025, sexes/ages = 24,155, salamanders/soil = 33,855). We also describe a core *E. e. xanthoptica* microbiome of bacterial OTUs that appeared in 90% or more of salamander samples. Patterns of diversity were then analyzed using the core_diversity_analyses.py script in QIIME [30]. The following alpha diversity metrics were calculated for all samples: OTU richness, Chao1, Faith's phylogenetic diversity (PD), and McIntosh evenness. We assessed whether alpha diversity metrics were normally distributed using Shapiro-Wilk tests.

Alpha diversity was compared among groups using analysis of variance (ANOVA) or Kruskal-Wallis tests in R (R Core Team, 2015). Beta diversity was calculated using weighted and unweighted UniFrac distances and Bray-Curtis dissimilarity in QIIME. Bacterial community composition between groups was compared using adonis in QIIME and plotted using principal coordinate analysis (PCoA) in R (R Core Team, 2015). We identified a core microbiome of OTUs that were present on at least 90% of all salamanders sampled.

We used the Antifungal Isolates Database developed by Woodhams et al. [34] to determine whether any OTUs present in the core microbiome matched bacterial species that have previously been isolated from amphibian skin and shown to have antifungal properties. To do so, we filtered our list of core microbiome OTUs by OTU ID to include only those that matched OTUs in the Woodhams et al. [34] database.

Results

Sequencing

Sequencing resulted in 11,512,105 reads representing 203,784 OTUs, most of which were represented by a single sequence. After filtering of all non-bacterial and low abundance reads, the final dataset included 4,912,877 reads representing 4398 OTUs across a total of 64 salamander and 20 soil samples.

Alpha Diversity

Our survey of 64 salamanders shows a large degree of variation in skin microbiome diversity across individuals. The number of OTUs (OTU richness) on individual salamanders ranged from 427 to 2423, while measures of McIntosh evenness ranged from 0.06 (community dominated by few taxa) to 0.94 (highly even community).

Measures of alpha diversity varied somewhat between populations, with East Bay generally exhibiting higher values than the other three populations. OTU richness, PD, and Chao1 were all normally distributed (Shapiro-Wilk $p > 0.05$), and McIntosh evenness was not (Shapiro-Wilk $p < 0.001$). ANOVA showed significant differences between the four populations in three measures of alpha diversity (OTU richness: $F = 7.03$, $df = 3$, $p < 0.001$; Chao1: $F = 5.897$, $df = 3$, $p = 0.002$; PD: $F = 5.658$, $df = 3$, $p = 0.003$). However, all four populations had similarly values of McIntosh evenness (Kruskal-Wallis $p = 0.868$). Tukey post hoc tests revealed that OTU richness was higher in the East Bay than all other populations (North Bay: $p = 0.044$; Sierra Nevada: $p < 0.001$; South Bay: $p = 0.011$). The East Bay population also had higher values of Chao1 and PD than the South Bay (Chao1: $p = 0.018$; PD: $p = 0.024$) and Sierra Nevada populations (Chao1: $p = 0.002$;

PD: $p = 0.002$), but the North Bay population was not different (Chao1: $p = 0.112$; PD: $p = 0.080$).

Within the North Bay population, Student's t test showed that all alpha diversity metrics were similar between sexes (OTU richness: $t(17.48) = 0.394$, $p = 0.698$; Chao1: $t(16.64) = 0.514$, $p = 0.614$; PD: $t(18.4) = 0.045$, $p = 0.965$; McIntosh evenness: $t(13.53) = -1.878$, $p = 0.082$). Three of the four alpha diversity metrics were also similar between adults and juveniles (OTU richness: $t(19.83) = 1.753$, $p = 0.096$; PD: $t(17.19) = 1.2875$, $p = 0.215$; McIntosh evenness: $t(25.06) = -0.817$, $p = 0.421$)), but adults had higher values of Chao1 than juveniles ($t(20.32) = 2.238$, $p = 0.036$).

Community Composition (Beta Diversity)

Using both unweighted UniFrac and Bray-Curtis dissimilarity metrics, we found that skin microbial communities differed between the four sampled populations (Fig. 2a, c; adonis unweighted UniFrac $p < 0.001$, $R^2 = 0.16547$; Bray-Curtis $p < 0.001$, $R^2 = 0.19448$). Individuals from the North Bay and Sierra Nevada populations harbored similar skin microbiomes to one another, while those of the East Bay population were significantly different. Individuals in the South Bay population showed variable microbiome community compositions; some were similar to the East Bay, and some were similar to the North Bay/ Sierra Nevada group (Fig. 2a, c). In contrast, the weighted UniFrac analyses showed no significant differences between any of the four populations (Fig. 2b, adonis $p = 0.258$).

Each population harbored a small set of bacterial taxa that were absent from the other populations, but these unique OTUs were all present at very low abundance (<0.01%, Supplemental Table 3). The East Bay had the most unique OTUs (112), while the other populations had few unique OTUs (14–20). None of these population-specific OTUs matched to any known amphibian mutualists present in the Woodhams et al. [34] database of bacteria with antifungal properties.

For the within-population analyses (North Bay), we found no difference in microbiome composition between males and females (adonis unweighted UniFrac $p = 0.492$; weighted UniFrac $p = 0.649$; Bray-Curtis 0.575). However, two of our diversity metrics revealed slight differences between adults and juveniles (adonis unweighted UniFrac $p = 0.084$ $R^2 = 0.04409$, Bray-Curtis 0.047 $R^2 = 0.06281$), while the third did not (weighted UniFrac $p = 0.451$) (Fig. 3).

In all populations, salamander skin microbiomes were highly distinct from microbial communities present in the surrounding habitat (soil) samples (adonis unweighted UniFrac $p < 0.001$, $R^2 = 0.10499$; weighted UniFrac $p < 0.001$, $R^2 = 0.32$; Bray-Curtis $p < 0.001$, $R^2 = 0.14331$) (Fig. 4). A number of OTUs were unique to either the soil or salamander datasets, but most were present in both soil and salamander samples (83% of OTUs identified).
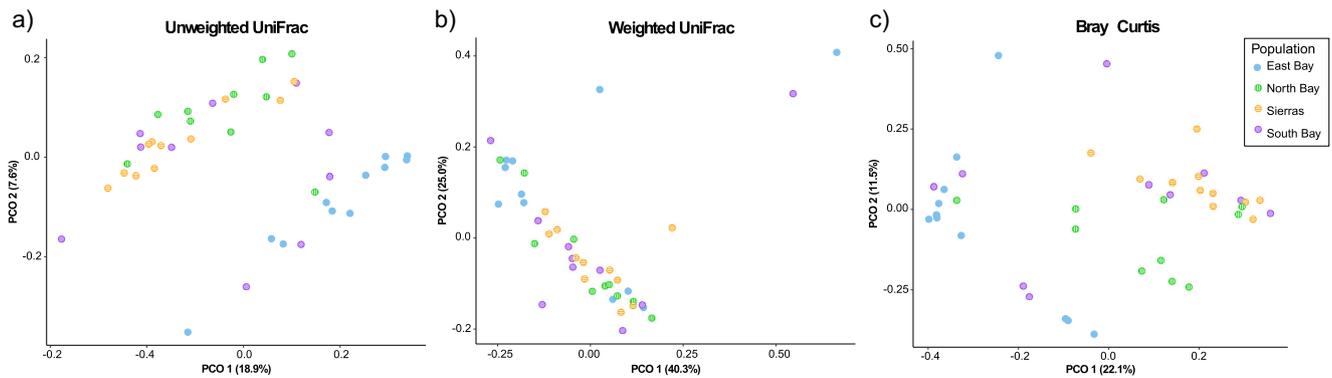
Fig. 2 Principal coordinate plots showing patterns of beta diversity across populations. Each *point* represents the microbiome of an individual salamander. Using **a)** unweighted UniFrac and **c)** Bray-Curtis, the skin microbial communities of the different populations are significantly distinct from one another. Using **b)** weighted UniFrac, the skin microbiomes of the four populations are indistinguishable

Core Microbiome

We identified a core community of 90 OTUs that were present in 90% or more of our salamander samples (Table 1). The core community dominated the skin microbiomes of most individuals, with an average relative abundance of 0.44 (minimum 0.03, maximum 0.82). Two individuals with low core community abundance (0.03 and 0.185, respectively) were dominated by OTUs of the family Chlamydiaceae.

Core OTUs were identified as belonging to the phyla Acidobacteria, Actinobacteria, Bacteroidetes, and Proteobacteria. The majority of core OTUs are members of the genus *Pseudomonas*, which account for an average relative abundance of 0.31 per salamander. One core OTU was identified as a member of the genus *Stenotrophomonas*, which has been shown to reside on amphibian skin and exhibit antifungal activity as well [32, 33].

Core community abundance did not differ significantly between populations (ANOVA $p = 0.1656$). Fifteen of the

90 core OTUs we identified matched bacterial isolates from the Antifungal Isolates Database [34]. Of these 15 OTUs, 10 were identified as *Pseudomonas* species and two more were unidentified species in the family Pseudomonadaceae, one was a *Microbacterium* species, and two were in the family Xanthomonadaceae (including *Luteibacter rhizovicinus*) (Table 1). Together, the 15 presumptive antifungal OTUs made up an average of 21.3% of the core microbiome across all individuals. Abundances of these OTUs were similar across populations (Kruskal-Wallis test $p = 0.6713$).

Discussion

In this study, we examined variation in skin microbiome community of the terrestrial salamander *E. e. xanthoptica*, a subspecies composed of four genetically and geographically distinct populations [28]. We characterized variation between
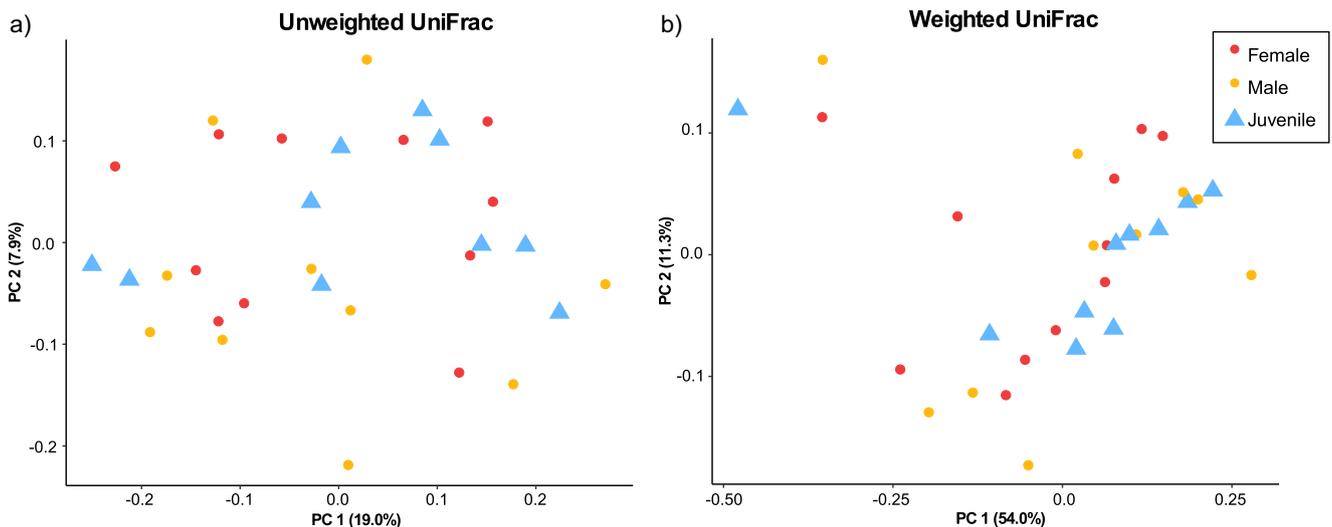

Fig. 3 Principal coordinates showing patterns of beta diversity between life history stages. **a)** Unweighted UniFrac and **b)** Weighted UniFrac. Unweighted UniFrac and Bray-Curtis analyses showed similar patterns, so Bray-Curtis are not shown

Fig. 4 Principal coordinates showing patterns of beta diversity between salamanders and habitat samples (soil). Unweighted UniFrac, weighted UniFrac, and Bray-Curtis analyses provided similar patterns, so only weighted are shown. Soil samples were taken beneath logs where salamanders were found. Salamander skin microbial communities were significantly different from soil microbial communities

Weighted UniFrac
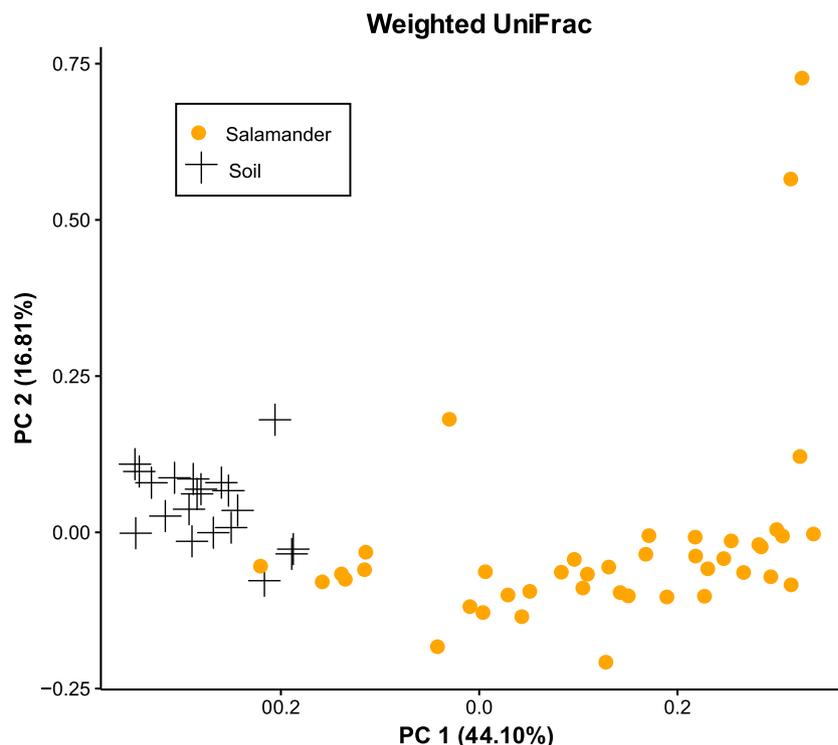
populations, ages, and sexes and compared salamanders to their habitats (soil) to determine if any of these factors are correlated with skin microbiome composition in this subspecies. We found that overall, individuals from the four populations of *E. e. xanthoptica* exhibit similar skin microbial communities, although each population also harbors a set of unique bacterial OTUs. Using two diversity metrics (unweighted UniFrac and Bray-Curtis), we found that the different salamander populations exhibited different microbiomes, but using the weighted UniFrac metric, these patterns disappeared (Fig. 2). Bray-Curtis and unweighted UniFrac distances are based on presence-absence of bacterial OTUs in each sample, while weighted UniFrac distances incorporate presence-absence as well as abundance of bacterial OTUs in each sample. Our results therefore suggest that the most abundant OTUs were similar across populations, but each population also harbored its own characteristic community, including a set of unique OTUs that were present at low abundances (<0.01%), the roles of which remain unknown (Supplemental Table 3). Microbiome communities have been shown to be highly dynamic, shifting composition between seasons, weeks, and even days [30, 35, 36]. It is thus possible for low abundance taxa to become abundant in response to changing environmental conditions or provide important functions even at low densities [37]. In the present study, it is unclear what role these rare or unique bacteria are playing in each population because none of the unique OTUs matched known amphibian mutualists.

Taken together, our analyses suggest that the dominant microbial taxa are relatively conserved across the four populations, but

the overall communities are nonetheless distinct. The overall similarity of the dominant microbes between the four sampled populations is striking and suggests that these taxa may play a role in host processes. These populations are separated by large distances (60–250 km) and have presumably been isolated from one another for hundreds to thousands of years [28]. If the skin microbiome was not tightly linked to host processes, we would expect to see differences arising over time in disjunct populations [38], as well as a higher degree of colonization from environmental microbes—yet, we found the opposite. In fact, the two salamander populations that had the most similar microbiomes were those that were furthest apart geographically—the North Bay and Sierra Nevada populations (Fig. 2a). Such a high level of similarity between the resident skin microbial communities in these populations suggests that the host-microbiome relationship has been conserved.

By contrast, the results of diversity-based analysis (Bray-Curtis and unweighted UniFrac) which showed differences in microbiome community across populations suggest that the patterns of similarity in dominant microbes do not fully capture the microbiome community dynamics. The differences in microbiome communities across populations (Fig. 2a, c) likely reflect a combination of factors. Each population differs from the others in environment, host evolution/genetic divergence, and stochastic processes, each of which could impact the microbiome community. For example, due to limitations caused by the significant drought in California throughout our sampling, individuals were found in different times of the year and experienced different climates, which might have influenced their

Table 1 Relative abundances of core OTUs for each population and life history stage

OTU_IDs	Taxonomy	Population				Life history group			
		East Bay	North Bay	Sierras	South Bay	Female	Male	Juvenile	Total
295031, 333782, 750018, 106985[a], 1566691[a], 552449, 831401[a], 277094[a], 633252, 589597[a] 541859[a] 646549, 398604, 108909, 280459, 350105, 202466[a], 780555, 669356, 4308187, 284090, 795272, 287032, 256834[a], 561294, 279231, 817734, 813617, 142419, 764682, 563273, 560886, 143131, 557974[a], 961783[a], New.ReferenceOTU5461, New.ReferenceOTU2542, New.ReferenceOTU5608, New.ReferenceOTU152, New.ReferenceOTU3451, New.CleanUp.ReferenceOTU4174133	Pseudomonas spp.	0.214503	0.350475	0.322958	0.238365	0.360858	0.338676	0.393682	0.317074
4378239, 624310, 1090029, 732609, New.ReferenceOTU1497	Sanguibacter spp.	0.018133	0.044374	0.055253	0.05018	0.035045	0.036346	0.060483	0.042831
537279, 160115[a], 544847	Family Xanthomonadaceae	0.034513	0.039326	0.025951	0.027777	0.030439	0.034771	0.052622	0.035057
338140, 544177, 829851[a], 818602[a]	Family Pseudomonadaceae	0.003024	0.015187	0.01669	0.008952	0.012721	0.015384	0.027199	0.014165
580625, 1105814	Family Bradyrhizobiaceae	0.007129	0.008705	0.008677	0.008268	0.010103	0.00941	0.00559	0.008269
884655, 1052559, 965129, 1003206	Family Enterobacteriaceae	0.00195	0.016151	0.006479	0.003776	0.00913	0.013283	0.003391	0.007737
1110763, 759061, New.ReferenceOTU3114	Sphingomonas spp.	0.012255	0.007255	0.00447	0.007523	0.007555	0.008393	0.006429	0.007697
459107[a], 348790, 814909, 1108350	Microbacterium spp.	0.004407	0.006815	0.00197	0.00415	0.005389	0.005936	0.008436	0.0053
281220, 196652, New.ReferenceOTU2127	Burkholderia spp.	0.006198	0.002566	0.00769	0.002298	0.005364	0.002915	0.002531	0.004223
546342, 848824	Family Sphingobacteriaceae	0.003176	0.003793	0.004861	0.001178	0.005711	0.004923	0.004803	0.004064
533999[a], 250626	Luteibacter rhizovicinus	0.004508	0.002413	0.00411	0.003469	0.005083	0.003806	0.002578	0.00371
876170, 279325, 1074625	Family Microbacteriaceae	0.006308	0.002527	0.003386	0.004333	0.003649	0.002999	0.0025	0.003672
391286	Stenotrophomonas spp.	0.000102	0.001893	0.006765	0.005451	0.001185	0.00115	0.001649	0.002599
1088265	Erwinia spp.	0.000729	0.002187	0.000463	0.002115	0.001834	0.008645	0.000482	0.00235
581021	Propionibacterium acnes	0.004875	0.002233	0.001343	0.004118	0.002147	0.000796	0.00043	0.002277

Average relative abundances of the 15 most abundant core taxa in order from highest to lowest abundance. The most abundant OTUs belonged to the genus Pseudomonas. Complete taxonomy and remaining OTUs are presented in Supplementary Table 2

[a] OTU with previously documented antifungal properties

microbiome communities. In addition, sampling for some populations was more concentrated geographically than other populations (Fig. 1). However, microbiome composition does not appear to be related to geographic spread of the individuals sampled; for example, the East Bay was sampled over a relatively small geographic area but had the highest OTU richness.

Such differences between populations could instead be potentially attributed to habitat differences. While all individuals were found in wooded areas, the vegetative communities were nonetheless different across populations, with East Bay individuals found primarily in suburban oak forest, South Bay populations in redwood forest, and North Bay and Sierra Nevada populations in mixed forests. While the limitations of our data set do not allow us to distinguish between these factors potentially impacting microbial community assemblage, it is nonetheless clear that microbial communities vary across populations but are still dominated by similar taxa, reflecting a combination of conservation of the host-microbiome relationship and variation in microbiome diversity across conspecific populations separated by significant geographic and temporal distances. These data therefore suggest that both host and environmental processes play a role in the maintenance of microbiome communities in this terrestrial salamander.

Core Microbiome

Identifying a core bacterial community is a key step towards understanding the relationship between host and microbiome. Core community taxa are shared across all members of the host group and are thought to perform ecological and physiological functions that influence host health and physiology [39].

We identified a core microbial community of 90 OTUs that were present in the 90% or more of our salamander samples (Table 1). The most abundant core OTUs belonged to the genus *Pseudomonas*, an ecologically diverse genus whose members include common species found in the environment, pathogens, and mutualists [40]. *Pseudomonas* is also one of the most common bacterial taxa in skin microbiomes of other amphibian species (e.g., [7, 41, 42]). Several other core OTUs in our dataset have also been found in other amphibian skin microbiomes, including *Sanguibacter* and *Microbacterium* [16, 40, 43], *Sphingomonas* [44], *L. rhizovicinus* [45], *Burkholderia* [41, 46], and members of the family Xanthomonadaceae (e.g., [24, 47, 48]). In addition, several of the core OTUs in our dataset are known from non-amphibian environments, such as soil and freshwater (*Achromobacter*, Xanthomonadaceae [49]) or plants and rhizospheres (*L. rhizovicinus*; [50]). Others are extremely widespread and have been found in a variety of environments (e.g., Bradyrhizobiaceae, Enterobacteriaceae, Sphingobacteriaceae, *Burkholderia*, *Stenotrophomonas*) [49]. It is unclear whether these bacterial taxa have any direct effects on the host or are simply commensal species that colonize amphibian skin. In

either case, their prevalence and abundance across the salamanders in our study suggest that they are important members of the skin microbiome community.

In addition to common environmental bacteria, we also identified a number of bacterial taxa in the core community that may be amphibian mutualists. For example, previous work has shown that many *Pseudomonas* species present on amphibian skin exhibit antifungal properties and likely aid in host defense against fungal pathogens [10, 12, 33, 51, 52]. Twelve *Pseudomonas* OTUs in our dataset matched bacteria that have previously been isolated from amphibian skin and tested for antifungal activity [34]. We also found one *Microbacterium* OTU and two OTUs in the family Xanthomonadaceae that matched antifungal isolates. We conclude that these taxa are therefore likely to be amphibian mutualists, aiding the host in combatting fungal pathogens such as *Batrachochytrium dendrobatidis*, the globally distributed agent of the disease chytridiomycosis [53]. The rest of the OTUs present in the core community did not match known antifungal species, but their prevalence and abundance in the majority of our samples suggest that they are likely selected for on salamander skin, forming either mutualistic or stable commensal relationships with the amphibian host. Even if these core bacterial taxa do not actively produce antifungal compounds on amphibian skin, they may nonetheless be playing a role in host health via alternative ecological mechanisms. For example, diverse communities are often better able to resist pathogens due to the dilution effect, in which the spread and maintenance of pathogens are inhibited by the diverse immune mechanisms and physiological properties of diverse hosts [54]. In addition, OTUs may generally be under negative-frequency-dependent selection, such that OTUs reaching high abundance are easily attacked by pathogens. In such a scenario, diverse communities would be more resistant to pathogens than low-diversity communities dominated by few species [55]. Thus, the diversity within the core community may aid in host pathogen resistance via any (or all) of the above mechanisms.

However, these results should be interpreted with caution. As described above, sequences were assigned to OTUs based on 97% similarity, but bacterial taxa with 97% or more similarity may exhibit different functional and ecological traits [56]. Thus, while sequences in our dataset did match sequences of previously identified antifungal bacteria, it does not necessarily mean the bacteria we sampled have the same capabilities. Conversely, bacteria that were not previously known to be antifungal may nonetheless have beneficial properties or functions for the host that have yet to be discovered [57]. Because neither *Ensatina* nor any other plethodontid salamander from the western USA has been surveyed before, it is possible that OTUs that did not match the antifungal database (for example, the OTUs we identified as unique to individual populations) are local mutualists that have not previously been detected. Further research into geographic variation in community composition and assessment of antifungal properties would shed light on this possibility.

While the skin microbiomes of most salamanders in the study were dominated by members of the core community, two individuals with low core community abundance were instead dominated by OTUs of the Chlamydiaceae family. To date, all known members of Chlamydiaceae are intracellular parasites and are known to cause disease in many animals [58]. Yet *Chlamydia* has rarely been recorded in amphibians, and its pathogenicity is unclear [59]. In any case, such low core community abundance and dominance of Chlamydiaceae may be an example of dysbiosis or disruption of a normal, healthy microbiome. Microbial dysbiosis is often associated with poor health or disease [40, 60], although it is not often clear whether dysbiosis leads to disease or is instead a symptom. Alternatively, it is possible that the Chlamydiaceae OTUs we observed represent minimally- or non-pathogenic variants that have not previously been documented. The individuals in our study that were dominated by Chlamydiaceae did not show any recognizable signs of disease, but observation time was limited (30 min per individual). In any case, we believe it is important to further explore the prevalence and effects of bacteria in the family Chlamydiaceae on amphibians to determine whether it is a potential threat to amphibian health.

Variation Across Life History Stages

Several studies have shown that skin microbiome composition shifts significantly during the transition from larval stage to adult in amphibians. It is hypothesized that such changes in the microbial community are caused by significant host physiological changes that coincide with a shift from aquatic to terrestrial environments, which themselves harbor significantly different microbial communities [7, 61]. Thus, changes in host physiology, environment, or both may contribute to shifts in skin microbiome composition, but it is not easy to tease these effects apart. *Ensatina e. xanthoptica* is a direct-developing species, and young emerge from the egg as terrestrial hatchlings, bypassing the larval stage [29]. Additionally, hatchlings live in the same habitats as adults. We therefore believe that this species provides an ideal system in which to test the influence of host age on skin microbiome composition, independent of significant metamorphic or ecological changes.

To that end, we examined variation between life history stages within one of our study populations (North Bay, Fig. 1) by comparing the skin microbial communities of equal numbers of juveniles, adult males, and adult females. Two of our community metrics showed that skin microbiomes of juveniles and adults were slightly different from one another (unweighted UniFrac and Bray-Curtis), while one (weighted UniFrac) did not (Fig. 3). The results of the weighted UniFrac analysis suggest that the dominant members of the skin microbiome community establish early in a salamander's life and are maintained into adulthood. The results of the unweighted UniFrac and Bray-Curtis analyses, which show slight but significant differences in composition between juveniles and adults, suggest that this process is not immediate and that either individuals may take time to achieve an optimal adult microbiome community or the optimal community may be different for juveniles and adults. In addition, the difference between adults and juveniles in our data is much more subtle than those shown by previous studies on metamorphic amphibians [7, 61], which suggests that the temporal stability of the amphibian skin microbiome may vary based on the host's life history strategy (metamorphic vs. direct-developing).

We also expected the two sexes to harbor different microbiomes, reflecting sex-based differences in physiology and behavior ([62, 63]). Research in other taxa has shown that the sex of the host may have a strong influence on microbiome
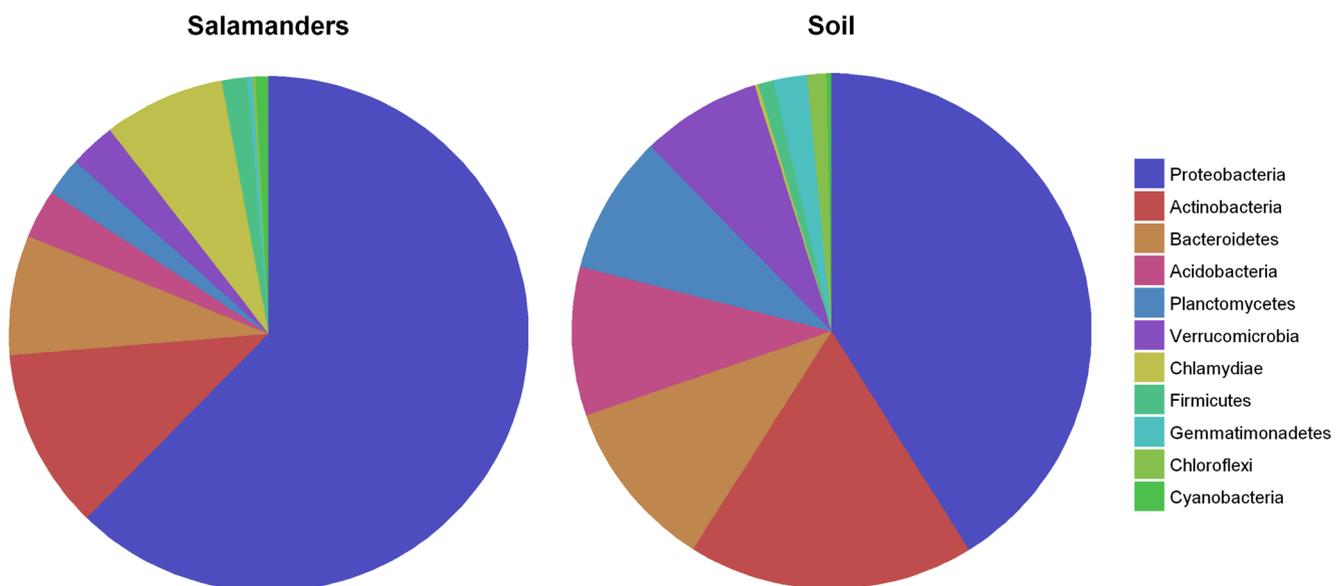

Fig. 5 Dominant bacterial phyla present on salamanders and in soil

composition [21–23], but to our knowledge, no study has yet investigated this in amphibians. In contrast to studies of other taxa, our data showed that male and female salamanders harbor similar microbiomes (Fig. 3), suggesting that host sex is not a determining factor for microbiome community composition in this species.

The similarity of skin microbiome composition across ages and sexes may have several explanations. For example, all individual salamanders within a population occupy similar habitats regardless of age or sex and are thus exposed to similar environmental conditions, potentially leading to similar skin microbiomes. Additionally, the similarity may be attributable to horizontal or vertical transmission of microbes [41, 64, 65]. *Ensatina* salamanders are known for their elaborate courtship behaviors, in which males and females engage in prolonged physical contact [29], which may serve to transfer and equalize microbial communities between the sexes. In addition, *Ensatina* also exhibit maternal care, in which the mother guards her eggs and hatchlings, maintaining nearly constant physical contact for at least several months [29]. This maternal contact may facilitate transfer of microbes to hatchlings, thus establishing the microbiome early in life [41, 66]. In either case, the similarity of microbiomes between ages and sexes suggests that a similar skin microbiome is cultivated and maintained throughout sexes and life stages, supporting the conclusion that the host-microbiome relationship is well conserved.

Impact of Environmental Microbes

Our final goal was to determine whether salamander skin cultivates a specific microbiome or is primarily colonized by microbes present in a salamander's habitat. In all four populations, salamander skin microbiomes were distinct from soil microbiomes (Fig. 4), but many of the OTUs present in these communities were not unique to one group or the other. Of the OTUs that were common to both groups, those that were highly abundant on salamander skin were not abundant in the soil and vice versa (Fig. 5). In agreement with previous studies [5, 7, 17, 57], this pattern suggests that while these different environments (soil and salamander skin) likely exchange bacteria, they each cultivate distinct community compositions.

Interestingly, we also observed a number of bacterial OTUs that were only present on salamander skin and were absent from the soil. There are several potential explanations for this finding. First, these OTUs may be environmental microbes that are either present in the soil at extremely low levels such that we were unable to detect them or they may originate from other areas of the salamander habitat that we did not sample, such as leaf litter or bark surfaces [16]. Alternatively, these OTUs may have coevolved with *E. e. xanthoptica*, being maintained through generations by horizontal or vertical transmission, thus originating not from the environment but from other salamanders [41, 67]. In either case, it is clear that the salamander skin microbiome does not simply mirror available soil microbes. Rather, it is likely that salamander skin selects for a specific bacterial community composed of environmentally available taxa as well as a possible suite of salamander-specific bacterial lineages.

Conclusions

Our study shows that individuals of four geographically and genetically distinct salamander populations harbor skin microbiomes that are dominated by a common core set of bacterial OTUs, but are nonetheless distinct from one another. Additionally, the skin microbiome appears to shift slightly over age classes but does not differ between sexes. These results suggest that the relationship between *E. e. xanthoptica* salamanders and their resident skin microbiomes is significantly influenced by both host and environmental processes.

Compliance with Ethical Standards

Ethical Approval All procedures performed with live animals in this study were approved by the Institutional Animal Care and Use Committee at San Francisco State University (Protocol no. A12-07). All sampling of wild salamanders was performed with approval from the California Department of Fish and Wildlife (SC-12920) and California State Parks.

Conflict of Interest The authors declare that they have no conflict of interest.

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