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The amphibian skin-associated microbiome across species, space and life history stages

JORDAN G. KUENEMAN,* LAURA WEGENER PARFREY,†§ DOUGLAS C. WOODHAMS,* HOLLY M. ARCHER,* ROB KNIGHT†‡ and VALERIE J. MCKENZIE*

*Department of Ecology and Evolutionary Biology, University of Colorado, Ramaley N-122, UCB 334, Boulder, CO 80309, USA, †Biofrontiers Institute, University of Colorado, Boulder, CO 80309, USA, ‡Howard Hughes Medical Institute, University of Colorado, Boulder, CO, USA

Abstract

Skin-associated bacteria of amphibians are increasingly recognized for their role in defence against pathogens, yet we have little understanding of their basic ecology. Here, we use high-throughput 16S rRNA gene sequencing to examine the host and environmental influences on the skin microbiota of the cohabiting amphibian species *Anaxyrus boreas*, *Pseudacris regilla*, *Taricha torosa* and *Lithobates catesbeianus* from the Central Valley in California. We also studied populations of *Rana cascadae* over a large geographic range in the Klamath Mountain range of Northern California, and across developmental stages within a single site. Dominant bacterial phylotypes on amphibian skin included taxa from Bacteroidetes, Gammaproteobacteria, Alphaproteobacteria, Firmicutes, Sphingobacteria and Actinobacteria. Amphibian species identity was the strongest predictor of microbial community composition. Secondarily, within a given amphibian species, wetland site explained significant variation. Amphibian-associated microbiota differed systematically from microbial assemblages in their environments. *Rana cascadae* tadpoles have skin bacterial communities distinct from postmetamorphic conspecifics, indicating a strong developmental shift in the skin microbes following metamorphosis. Establishing patterns observed in the skin microbiota of wild amphibians and environmental factors that underlie them is necessary to understand skin symbiont community assembly, and ultimately, the role skin microbiota play in the extended host phenotype including disease resistance.

Keywords: amphibians, bacteria, California, metamorphosis, microbiome, skin

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Introduction

Viewing animals as habitats for microbial communities informs and expands our understanding of the roles microbes contribute to host functions. Recent studies show that microbial symbionts of animals play many important roles (McFall-Ngai *et al.* 2013), yet the symbionts of most hosts are understudied, and basic ecological principles of host-associated community assembly remain unknown for all but a few species. Understanding these complex ecological and evolutionary patterns and processes across species and scales remains a challenge, and many taxa and habitats must be studied to infer general principles. An increasing number of animal microbiomes are becoming sequenced; however, most studies to date focus on the mammalian gut (Ley *et al.* 2008; Muegge *et al.* 2011; McFall-Ngai *et al.* 2013). In this context, our investigation of amphibian skin communities provides a unique opportunity to rigorously and nondestructively sample an underrepresented microbiome in a threatened host taxon.

Animal skin provides a physical barrier against moisture loss, invasion of pathogens and exposure to harmful

Correspondence: Valerie McKenzie, Fax: 303 492 8699;
E-mail: valerie.mckenzie@colorado.edu
§Present address: Departments of Botany and Zoology, University of British Columbia, Vancouver, BC, Canada

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chemicals and also hosts a consortium of microbiota that provide services such as vitamin synthesis and pathogen defence (Grice & Segre 2011; Rosenthal *et al.* 2011). In addition to diverse bacteria, human skin harbours fungi, viruses, unicellular eukaryotes and micro-arthropods all interacting with each other and with the host's immune system in the skin matrix (Grice & Segre 2011). Thus, the skin is a complex ecosystem shaped by interactions with the environment and host factors that influence colonization and resilience dynamics (Costello *et al.* 2009; Rosenthal *et al.* 2011).

Amphibian skin is a particularly complex organ involved in processes including respiration, osmoregulation, thermoregulation, pigmentation, chemical communication and pathogen defence (Duellman & Trueb 1994; Campbell *et al.* 2012). The mucous layer of amphibian skin, rich in glycoproteins, harbours many microbial symbionts and pathogens (Austin 2000). Species-specific oligosaccharides are thought to mediate specific microbial interactions at all amphibian life stages from egg to adult (Varki 1993; Delplace *et al.* 2002). Components of the innate and adaptive immune systems, including lysozymes, antimicrobial peptides and mucosal antibodies, add to the complexity of this unique ecosystem (Rollins-Smith & Woodhams 2012). These skin properties and components may interact with microbial inputs from the host's environment to help mediate colonization of the skin.

Amphibian skin is among the best-studied systems for understanding skin-associated microbial communities. Much of this research has been culture-based, targeted at *Batrachochytrium dendrobatidis* (*Bd;* Bletz *et al.* 2013*)*, the panzootic fungal pathogen that has decimated amphibians cross-continentally (Kilpatrick *et al.* 2010), and has demonstrated that amphibian skin microbial communities mediate disease susceptibility (Woodhams *et al.* 2007; Harris *et al.* 2009). Infectivity and severity of disease in susceptible amphibians have been reduced via antifungal compounds produced by particular bacteria (Bletz *et al.* 2013). Thus, investigation of the basic ecology that structures skin microbiota on amphibians will inform the design and application of microbial therapeutics, which is an active area of research for mitigating *Bd*. Using 16S rRNA gene sequencing, McKenzie *et al.* (2012) found that species identity was a strong driver of amphibian skin bacterial communities. However, this study did not span a broad geographic region or different life stages, which have been shown to be important for influencing other microbial communities such as in the human gut (Yatsunenko *et al.* 2012).

Culture-independent approaches allow further insight into the amphibian skin microbiome, allowing identification of unculturable members of the community and addressing which factors are most important in structuring the amphibian skin microbiome as a whole. Additionally, the influence of abiotic environmental factors on host-associated microbial communities is an underexplored topic that may become increasingly relevant with environmental change (Belden & Harris 2007). Previous studies have found factors such as pH, salinity and temperature to be the primary predictors of microbial communities in host and nonhost-associated environmental samples (Fierer & Jackson 2006; Lozupone & Knight 2007; Costello *et al.* 2009; Ritchie 2011). Consequently, properties of the freshwater environment might affect microbes on the skin, which is in constant contact with the environment and undergoes a continual process of microbial exchange.

Sampling wild amphibians and their environments allows us to differentiate between host and environmental drivers of amphibian skin communities, and overcome several sampling challenges associated with studies in humans or laboratory specimens. Cohabiting, premetamorphic amphibians occupy the same environmental niche during their larval development and will mature within a single pond. Consequently, they have a known environmental origin and a shared history among individuals and species. Amphibians that newly metamorphose from ponds have very limited dispersal and share influences on the microbiome, such as climate, water quality, diet, plants and other organisms, which may modulate colonization of the skin by microbiota. Importantly, different ponds that harbour multiple species of amphibians can serve as replicates to examine patterns of host-associated microbial community assembly across different sites (McKenzie *et al.* 2012). Here, we compare and contrast microbial skin communities of amphibian species with each other and with their corresponding environments.

This study investigates natural diversity and distribution of skin microbes across wild amphibian populations from central and northern California to answer three key questions. First, is the composition and relative abundance of microbial communities on amphibian skin species-specific? Second, do populations of amphibians have unique microbial communities across sites they inhabit, and are those differences linked to abiotic factors? Third, do microbial communities vary across cohabiting life history stages within one amphibian species, *Rana cascadae*? Given the dramatic physical and biochemical changes that occur during amphibian metamorphosis, such as immune system development and keratinization and hardening of the skin tissue, we might expect microbial communities to also change substantially during this process (Rollins-Smith and Woodhams 2012). Examination of the types of natural variation found in skin-associated microbial communities of amphibians will

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inform applied amphibian conservation by advancing knowledge about microbial targets for cultivation and how they are distributed in natural populations. We view these questions as building a basic ecological foundation to better understand the role of microbial communities in host–pathogen defence and to enhance understanding of how microbial therapies may be applied to threatened amphibian species in future trials.

Methods

Field sampling

During June through August of 2011, we sampled amphibians from two regions: Joseph Grant Park in California's Central Valley, and the Trinity Alps in Northern California (Fig. 1). Wetlands in the Central Valley region were surveyed for cohabitating amphibians (*Anaxyrus boreas*, *Pseudacris regilla*, *Taricha torosa*, *Lithobates catesbeianus*), and lakes in Northern California were surveyed for *Rana cascadae* populations to explore amphibian microbial communities across larger geographic areas and to capture developmental stages from larvae to adults. Amphibians from a given site were all collected on the same day. Sampling location and sample size of each amphibian and life history stage are given in Table 1. In Table 1, the term 'metamorph' indicates an individual that has recently transitioned from the tadpole stage (Anura) or larval stage (Caudata) within the sampling season, and is now a smaller version of an adult (legs, no gills, etc.). Metamorphs may still retain a slight tail but are a year from becoming subadults and often 2–3 years from becoming sexually mature adults, depending on species. For clarity of analyses here, adult and subadult and metamorph are grouped into postmetamorph and tadpoles and larvae are described as pre-metamorph. The term subadult is used for *Rana cascadae* to distinguish between reproductive adults and nonreproductive subadults postmetamorphosis. Permits and authorization were granted by California Fish and Game, East Bay Parks, and the University of Colorado IACUC.

All amphibians were captured using a dip net, and each individual was handled with new nitrile gloves. Prior to specimen sampling, each individual was rinsed with 50 mL of sterile water two times to ensure that the skin sample primarily included skin-associated microbes rather than pond-associated material, including pond

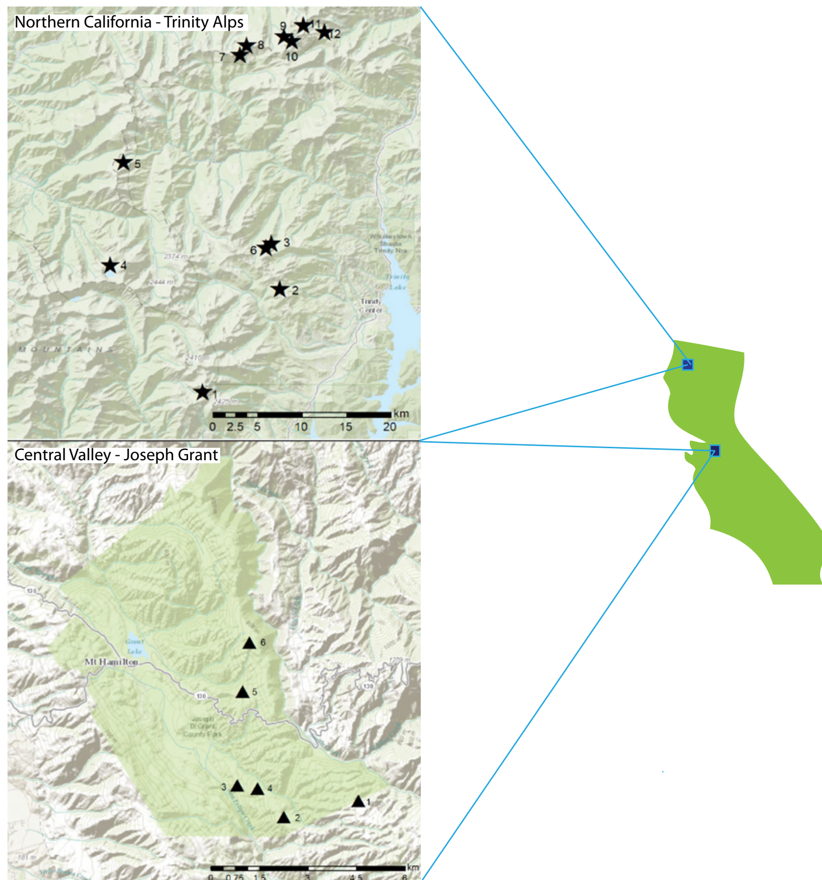

Fig. 1 Map of amphibian sampling areas: Joseph Grant Park, California's Central Valley and the Trinity Alps, in Northern California. Numbers correspond to sites described in Table 1.

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Table 1 Samples included from each region, site, amphibian species and life history stage. Tadpoles were samples at developmental stage 39 (Gosner 1960). Subadult *Rana cascadae* are 2nd year metamorphic individuals that are not reproductively mature, versus adults that are 3 years or older and reproductively mature

Site	Species	Life history stage	Total (*N*)
Central Valley – Joseph Grant			
1. Manzanita	*Anaxyrus boreas*	Metamorph	8
	Pseudacris regilla	Metamorph	7
	Lithobates catesbeianus	Tadpole	8
2. Eagle Lake	*Anaxyrus boreas*	Metamorph	8
	Pseudacris regilla	Metamorph	4
3. Rattlesnake	*Anaxyrus boreas*	Metamorph	8
	Pseudacris regilla	Metamorph	4
4. No Talk	*Anaxyrus boreas*	Metamorph	8
	Pseudacris regilla	Metamorph	8
	Lithobates catesbeianus	Tadpole	3
	Taricha torosa	Larvae	8
5. Yerba Buena	*Anaxyrus boreas*	Metamorph	8
	Taricha torosa	Larvae	8
6. Krammer	*Anaxyrus boreas*	Metamorph	7
	Pseudacris regilla	Metamorph	7
Northern California – Trinity Alps			
1. Echo	*Rana cascadae*	Adult	3
2. Shimmy/ Little Shimmy	*Rana cascadae*	Adult (4) Subadult (3)	7
3. Tapie	*Rana cascadae*	Adult	6
4. Little Caribou	*Rana cascadae*	Adult	4
5. Adams	*Rana cascadae*	Adult	4
6. Found	*Rana cascadae*	Adult (6) Subadult (11)	17
7. Section line	*Rana cascadae*	Adult (12) Subadult (8) Tadpoles (4)	24
8. Mavis	*Rana cascadae*	Adult	5
9. Middle Boulder	*Rana cascadae*	Adult (2) Subadult (3)	5
10. 26186	*Rana cascadae*	Adult	6
11. 26184	*Rana cascadae*	Adult	8
12. Little Marshy	*Rana cascadae*	Adult	2

water, sediment and transient microbes (Culp *et al.* 2007; Lauer *et al.* 2007; McKenzie *et al.* 2012). Earlier studies by Culp *et al.* (2007) and Lauer *et al.* (2007) demonstrated that the composition of bacteria obtained from amphibian skin versus rinse water differ substantially, suggesting that most of the bacteria observed in this study are associated with the amphibians and not transient bacteria from the environment. Immediately following rinsing, each amphibian was sampled using a sterile cotton-tipped swab brushed over the entire ventral surface and limbs of the amphibian for 30 s. For larvae, the entire body was uniformly swabbed following the protocol of McKenzie *et al.* (2012). All sampling was nondestructive, and individual amphibians were released within 15 min to the site of capture. Environmental water samples were collected by moving a sterile swab through the water for 30 s at a depth of approximately 40 cm. Sediment samples were collected at the same location as water samples by embedding the swab into the sediment for 30 s. Soil samples were collected by removing 2 g of soil from the top 2 cm, within a metre of the pond's edge, using a sterile swab. All environmental sample types were collected as close as possible to where amphibians were captured and sampled. Each swab was placed in a sterile vial and stored on ice for transfer to a −20 °C freezer for storage until DNA extraction. At each site, abiotic water quality measurements were taken at the time of amphibian sampling using a YSI multiprobe field instrument. Data recorded from the YSI included temperature, pH, specific conductivity (a measure of how well water can conduct an electrical current measured in microSiemens per centimetre), oxidation–reduction potential and dissolved oxygen. A distance-based linear model (DistLM) analysis was performed to assess correlations between measured water quality factors and amphibian skin microbial communities using PRIMER 6. Models incorporating various combinations of factors were compared by Akaike's information criteria, and the best model obtained separately for Northern California and Central Valley sites, respectively.

DNA extraction/sample processing

DNA extraction was performed utilizing the MoBio Power Soil Extraction kit (MoBio Laboratories, Carlsbad, CA, USA). The standard MoBio protocol was used with minor adjustments for samples with low DNA amounts, described in detail in Fierer *et al.* (2008). Adjustments included incubating samples in 65 °C after the addition of C1, vortexing the PowerBead tubes horizontally for 2 min, and allowing solution C6 to sit on the filter for 5 min before the final elution (Lauber *et al.* 2008). Each 25 μL PCR contained: 11 μL PCR water, 10 μL 5 Prime Master Mix, 1.0 μL each of the forward and reverse primers (0.4 μM final concentration), 1.0 μL $MgCl_2$ and 1.0 μL genomic DNA. PCR primers (F515/R806) were used to target the V4 region of 16S rRNA, the reverse PCR primer contained a 12 base error correcting Golay barcode as described in Caporaso *et al.* (2011). PCR conditions were comprised of a denaturation step of 94 °C for 3 min, followed by 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s and final extension at 72 °C for 10 min. The PCR was

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performed in triplicate and combined after amplification giving a total volume of approximately 75 μL. Amplicons were then quantified using the Quant-IT Picogreen dsDNA reagent. Samples were pooled into one sample per plate by combining equal concentrations of each amplicon. These pools were then cleaned using the MoBio UltraClean PCR clean-up DNA purification kit. Following clean-up, samples were again quantified using PicoGreen reagent and equal concentrations were pooled into one final pool for sequencing. A NanoDrop spectrophotometer was used to determine the purity and DNA concentration of this pool. Finally, the pool was sequenced using an Illumina HiSeq 2000 instrument at the BioFrontiers Institute Next-Generation Genomics Facility at the University of Colorado, Boulder.

Sequence analyses

Amplicons were sequenced on 1/3 of an Illumina HiSeq lane at the University of Colorado, Boulder, yielding 100 bp reads. QIIME v1.6.0 (Caporaso *et al.* 2010) was used for sequence analysis unless otherwise noted. Sequences were filtered for quality and assigned to their respective sample using default settings. The resulting 33.4 million sequences were clustered into operational taxonomic units (OTUs) at 97% similarity according to the subsampling open-reference protocol (http://qiime.org/tutorials/open_reference_illumina_processing.html) using the October 2012 version of the Greengenes reference database (greengenes.secondgenome.com; McDonald *et al.* 2012; DeSantis *et al.* 2006). Of the 33.4 million sequences, 83% matched sequences in the reference database and the remaining 17% of the sequences were clustered into de novo OTUs with UCLUST (Edgar 2010) according to the open reference protocol. OTUs with fewer than 100 reads and those present in only one sample were filtered out of our analysis according to recommendations from Bokulich *et al.* (2013), resulting in a total 31.1 million sequences clustered into 9602 unique OTUs. OTUs that matched the Greengenes reference database inherited the Greengenes taxonomy, and taxonomy was assigned to the de novo OTUs using the RDP Classifier (Wang *et al.* 2007) with an 80% confidence threshold. Additionally, we placed the sequences of several abundant de novo OTUs into the Greengenes reference tree using the RAxML EPA algorithm (Berger *et al.* 2011) to better assess their phylogenetic relationships and gain more detailed taxonomic assignment. Sequences were aligned to the Greengenes reference alignment using PyNAST (Caporaso *et al.* 2010), and a tree was constructed with FastTree (Price *et al.* 2010) according to standard procedures within QIIME. Samples with fewer than 19 000 sequences per sample, including experimental and sequencing controls, were removed from the analysis, yielding 227 samples in the final data set: 195 amphibian samples and 32 environmental samples. Analyses were conducted on data rarefied to 19 950 sequences per sample. The following alpha diversity metrics were calculated: richness, Chao1, PD whole tree (phylogenetic diversity) and Shannon and Simpson diversity indices. Alpha diversity was compared among groups by analysis of variance or Kruskal–Wallis tests in IBM SPSS Statistics 21. Nonparametric tests were used when data transformations could not correct for unequal variances among treatments (Levene's test). Beta diversity was calculated within QIIME using the unweighted UniFrac metric (Lozupone & Knight 2005). The resulting distance matrix was imported into PRIMER 6 for further analysis. The relative contribution of host species, sample site and amphibian age was statistically analysed using a PERMANOVA (PRIMER 6) and plotted using principal coordinates analysis (PCoA). We assessed the association between specific OTUs and host species using ANOVA tests comparing samples for one species to all remaining samples within QIIME (qiime.org/scripts/otu_category_significance.html). To correct for the large number of comparisons that result from the large number of OTUs, we used FDR-corrected *P*-values.

Controls included 13 sterile rinse water samples and 3 glove samples. Although very few sequences were recovered from these samples, 27 OTUs were present in two or more of these samples. These OTUs were considered potential contaminants. Analyses of beta diversity including or omitting these OTUs did not influence the patterns or statistical results reported here. For taxonomic comparisons, these OTUs were removed. The most abundant, or dominant, OTUs found in amphibians and environmental samples that had a relative abundance of >3% of sequences within each species were examined for similarities among sample types. Rarefaction plots of phylogenetic alpha diversity were created to compare sampling completeness and alpha diversity among species, life history stages and environmental samples. Rarefaction was performed at 8000 sequences per sample to include the majority of samples and for simple visual interpretation.

To address our first question pertaining to host species effects on amphibian skin-associated bacteria, we examined the bacterial sequences from the skin of four cohabiting amphibian species at six sites in the Central Valley: Eagle Lake, Krammer, Manzanita, No Talk, Rattlesnake and Yerba Buena (Table 1; Fig. 3). Environmental samples from these sites (lake water and soil) are included in the PCoA (Fig. 3) for comparison with amphibian samples, but are not included in the ANOSIM analysis assessing the relative importance of host species versus site. To examine host species and site as

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predictors of amphibian skin-associated bacteria, we performed a two-factor crossed ANOSIM with host species and sample site as factors for all four species sampled in the Central Valley (Table 1). We also refined this comparison using a nonparametric MANOVA in PAST version 2.16, to test host and site as predictors of amphibian skin-associated bacteria of early metamorphs of two species (*A. boreas and P. regilla*) in the Central Valley, thus controlling for developmental stage. To evaluate our second question, the role of environment in structuring amphibian microbial communities, we isolated the effect of site by comparing samples within each species observed across multiple sites, respectively: *P. regilla* at five sites, *A. boreas* at six sites, *T. torosa* at two sites and *R. cascadae* at 12 sites. *Lithobates catesbeianus* was excluded from our analysis of site effects due to low sample size. To assess our third question concerning the effects of developmental life stage on the skin-associated microbial communities, we sampled amphibians from different life stages at one site (Section Line Lake). Longitudinal studies of Cascades frogs have occurred at this location for longer than 5 years (Piovia-Scott *et al.* 2011), indicating the species has occupied this site successfully for a significant time period.

Results

In our evaluation of 195 amphibian and 32 environmental samples, we found significant differences among species, life history stages and environmental samples in alpha diversity (richness, Chao1, Shannon diversity index, Simpson diversity index and phylogenetic diversity; Kruskal–Wallis test, all $P < 0.001$; Fig. 2). The highest levels of alpha diversity were found in soil, sediment and subadults of *A. boreas*. Tadpoles of *R. cascadae* had the lowest phylogenetic diversity, and both *R. cascadae* tadpoles and *L. catesbeianus* had the lowest Shannon diversity index. Lake water and all other amphibian species and age groups analysed had intermediate diversity (Fig. 2). Dominant OTUs were unequally represented across species and life history stages and were different than those detected in lake water, sediment or soil (Fig. 4). Subadult and adult *R. cascadae* had abundant representation of the family Comamonadaceae and a similar composition of dominant OTUs, whereas bacterial communities of *R. cascadae* tadpoles were unique compared with post-metamorphic stages. We observed a disproportionate amount of *Pseudomonas* on *R. cascadae* tadpoles, and this genus was also common in lake water. Three dominant OTUs found in high abundance in water belonged to the genera *Ramlibacter*, *Leptothrix* and *Pseudomonas*.

The Central Valley data set was used to test the relative importance of host species versus environment, as up to four cohabiting species were sampled at six sites (Fig. 3). Host species was the best predictor of skin bacterial community similarity, but sample site also explained a significant proportion of the variation (ANOSIM analysis of the unweighted UniFrac distances with factors host species

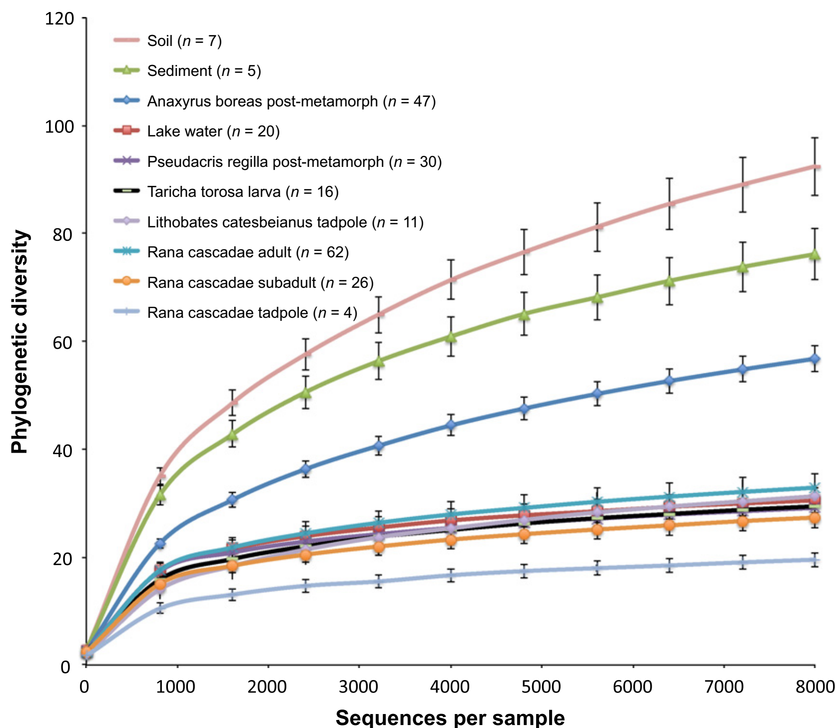

Fig. 2 Plot comparing alpha diversity of amphibian species, life history stages and environmental samples. Mean phylogenetic diversity (±SE) of each sample type (e.g. across individuals of a species at all sites) captured across sequencing depths.

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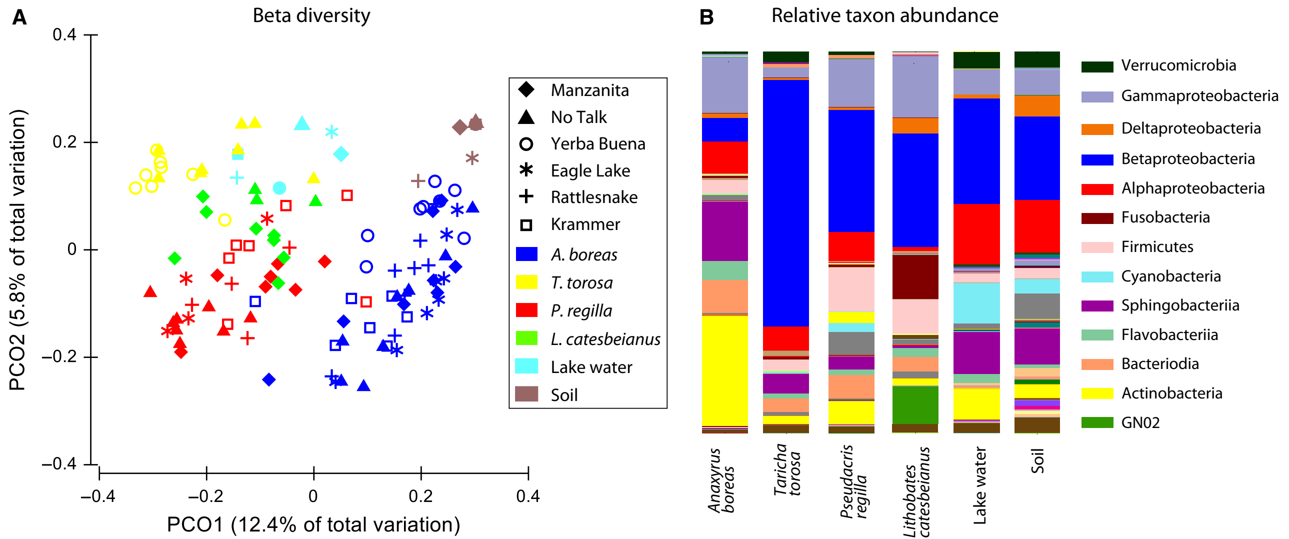

Fig. 3 Microbial communities associated with amphibian skin are differentiated across host species in the Central Valley. (A) Principal coordinates analysis plot of unweighted UniFrac distances for four amphibian species sampled from six sites. Each point represents the skin microbial community of an individual amphibian; colour indicates species; Dark Blue = *A. boreas*, Yellow = *T. torosa*, Red = *R. regilla*, Green = *L. catesbeianus*, Light blue = Lake water, Brown = Soil. Shape indicates pond locations: closed square = Manzanita, triangle = No Talk, circle = Yerba Buena, star = Eagle Lake, cross = Rattlesnake, open square = Krammer. Not all species are sampled from each site (see Table 1). Samples included are from postmetamorphs of *A. boreas* (N = 47) and *P. regilla* (N = 30), larvae of *T. torosa* (N = 16) and larvae of *L. catesbeianus* (N = 11). Environmental samples include lake water and soil from each site. (B) The relative abundance of sequences assigned to major bacterial taxa in our data set (pooled per species). See Fig. S1 (Supporting information) for plots of the relative taxonomic abundance for individual samples.

and site; species $R^2 = 0.761$, $P = 0.001$; site $R^2 = 0.456$ $P = 0.001$). When refined to include only the early metamorphic life stage (*P. regilla* and *A. boreas*), host species explained more variation of the bacterial community (two-way nonparametric MANOVA $F_{1,65} = 70.285$, $P = 0.0001$) than site ($F_{5,65} = 6.0509$, $P = 0.0001$), and the interaction of species and site was not significant ($F_{5,65} = -2.3699$, $P = 0.9965$). The differences between species were driven by broad differences in the relative sequence abundance of phylum and class-level taxa (Figs 3B, 4). Dominant bacterial taxa differed among species, and all four species harboured one or a few host-specific OTUs comprising 8–25% of the overall community (Table S1, Supporting information). For example, Fusobacteria were abundant only on *L. catesbeianus* individuals (Fig. 3B; Table S1, Supporting information) and nearly all sequences correspond to an uncultured genus in the Fusobacteriaceae that is significantly associated with *L. catesbeianus* (ANOVA $P < 0.001$, Table S1, Supporting information). *Anaxyrus boreas* had abundant Actinobacteria (Fig 3B; Table S1, Supporting information), 25% of the reads for *A. boreas* samples in the Central Valley belonged to one de novo OTU that is a close sister group to the genus *Gardnerella* (as assessed by the RAxML placement tree, Table S1, Supporting information). The association of this OTU with *A. boreas* was highly significant (ANOVA $P < 0.001$, Table S1, Supporting information) and it was found on all individuals. *Gardnerella* was also found at low abundance (approximately 2% abundance) on *P. regilla* (Table S1, Supporting information).

Because bacterial communities were found to be host species specific, the effect of site was analysed within each species that was sampled from multiple pond sites using ANOSIM and ADONIS. Indeed, sample site had a significant effect on the skin-associated bacteria of all species: *P. regilla* (N = 30 metamorphs from five sites), ANOSIM $R^2 = 0.554$, $P = 0.001$, ADONIS $R^2 = 0.101$ $P = 0.001$; *A. boreas* (N = 47 metamorphs from six sites), ANOSIM $R^2 = 0.435$, $P = 0.001$, ADONIS $R^2 = 0.217$ $P = 0.001$; *T. torosa* (N = 16 larvae from two sites), ANOSIM $R^2 = 0.45$, $P = 0.001$, ADONIS $R^2 = 0.125$, $P = 0.001$; *R. cascadae* (N = 88 postmetamorphs from 12 sites), ANOSIM $R^2 = 0.451$, $P = 0.001$, ADONIS $R^2 = 0.237$, $P = 0.001$ (Fig. 5).

We found that soil and lake water had many shared OTUs with the amphibians sampled in the Central Valley (Soil = 45–62%, Lake water 76%; Lake water shared 26–41 per cent of OTUs with amphibians and soil, Table S2a,b, Supporting information). One manifestation of this pattern is the observed similarity between the terrestrial *Anaxyrus* and soil samples and the predominately aquatic *Pseudacris* and lake water (Fig. 3A). Yet, amphibian skin communities of each species are distinct from the environmental samples (ANOSIM amphibian vs. lake water and soil: *Anaxyrus*: $R^2 = 0.641$; $P = 0.001$;

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OTU ID	Taxon	Development			Host				Environment		
		Rana cascadae adult (n = 62)	*Rana cascadae* subadult (n = 26)	*Rana cascadae* tadpole (n = 4)	*Lithobates catesbeianus* pre-metamorph (n = 11)	*Taricha tarosa* pre-metamorph (n = 16)	*Pseudacris regilla* post-metamorph (n = 30)	*Anaxyrus boreas* post-metamorph (n = 47)	Lake water (n = 20)	Sediment (n = 5)	Soil (n = 7)
1898675	*Ramlibacter sp.*	0.30	1.20	0.00	0.00	0.00	0.03	0.02	8.81	0.36	0.12
258496	*Leptothrix sp.*	0.51	1.84	0.01	0.00	0.00	0.04	0.01	5.45	0.18	0.01
293741	*Pseudomonas sp.*	1.32	0.10	19.40	0.15	0.05	0.21	2.32	4.50	0.02	0.01
70346	f__Chitinophagaceae	0.09	0.03	0.00	0.00	3.45	0.00	0.01	0.94	0.04	0.03
348567	f__Comamonadaceae	0.02	0.11	0.02	0.00	3.48	0.22	0.08	0.09	0.01	0.06
533038	f__Methylophilaceae	0.00	0.00	0.00	0.00	3.85	0.02	0.05	0.03	0.02	0.03
537871	f__Enterobacteriaceae	0.04	0.09	0.04	7.08	0.05	0.65	1.28	0.03	0.00	0.02
532752	f__Fusobacteriaceae; g__u114	0.02	0.00	0.00	15.64	0.23	0.18	0.04	0.02	0.01	0.31
72607	f__Comamonadaceae	0.00	0.00	0.00	0.00	15.02	0.00	0.00	0.01	0.00	0.00
320198	*Methylotenera sp.*	0.00	0.00	0.00	23.84	0.01	0.01	0.01	0.01	0.00	0.00
de novo 480633	p__Bacteroidetes	13.22	8.14	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00
752012	*Dysgonomonas sp.*	0.00	0.00	8.44	0.00	0.00	0.00	0.04	0.00	0.00	0.00
632140	*Sphingobacterium multivorum*	0.00	0.03	0.00	0.00	0.00	0.08	5.68	0.00	0.00	0.01
562181	f__Alcaligenaceae	0.00	0.00	0.00	0.00	0.00	20.31	0.07	0.00	0.00	0.00
245980	o__Streptophyta	0.18	0.02	0.00	0.03	0.02	5.38	0.08	0.00	0.00	0.00
190913	Bacteroides sp.	0.02	0.03	0.00	0.01	0.01	1.11	3.85	0.00	0.00	0.00
2450505	Bacteroides sp.	0.30	0.18	8.78	0.00	0.14	0.40	0.77	0.00	0.00	0.00
de novo 541501	Novel Proteobacteria	0.00	0.00	0.00	5.81	0.00	0.00	0.00	0.00	0.00	0.00
442126	Sphingobacterium sp.	0.00	0.00	0.00	0.00	0.00	0.02	3.33	0.00	0.00	0.00
de novo 480582	Novel bacterium	0.00	0.00	0.00	5.74	0.84	0.01	0.00	0.00	0.00	0.00
de novo 508406	Novel bacterium	0.00	0.00	5.79	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Fig. 4 Heatmap comparing the relative abundances of selected operational taxonomic units (OTUs) across life history stages of *R. cascadae*, other amphibian species and environmental samples. OTUs with an average relative abundance of >3% of total sequences were selected from each species/life history stage and from lake water.

Taricha: R^2 = 0.806 P = 0.001; *Pseudacris*: R^2 = 0.443 P = 0.001; *Lithobates*: R^2 = 0.623 P = 0.001). Differences among dominant OTUs can be visualized in Fig. 4. Across the northern California sites, *Rana cascadae* skin communities also differ significantly from environmental samples (ANOSIM R^2 = 0.292 P = 0.001; amphibian N = 88, environmental samples N = 19).

Distance-based linear modelling with stepwise AIC performed on water quality measurements indicated that specific conductivity was the only significant factor correlated with amphibian microbiota for the Central Valley, though it explained only 3% of overall variation. The analysis for sites in Northern California identified a model that included oxidation–reduction potential, specific conductivity, temperature, pH and dissolved oxygen as significant factors. Together, these factors explained only 11% of the variation in the skin bacterial communities although the effect is highly significant (DistML P = 0.001).

At Section Line Lake, we sampled *R. cascadae* from all major life history stages present during the time of sampling, including tadpoles, subadults and adults (Fig. 6). In addition, we sampled lake water and sediment to try and identify overlap between amphibians and their environment. We found no differences between postmetamorphic members of this population (ANOSIM of subadults, N = 8 vs. adults, N = 12, R^2 = –0.078 P = 0.85). We do detect a significant separation of tadpoles with respect to all postmetamorphic stages sampled (ANOSIM of tadpoles, N = 4 vs. postmetamorphic individuals, N = 20, R^2 = 0.828, P = 0.001) and find tadpoles to be less similar to lake water than postmetamorphic amphibians (Fig. 5). The postmetamorphs at Section Line are characterized by very high abundance, more than 65% overall, of one OTU corresponding to the family Comamondaceae (ANOVA P < 0.001, Table S3, Supporting information). In contrast, tadpoles harboured high levels of Pseudomonads and Bacteroidetes in the genera *Bacteroides* and *Dysgonomonas* (ANOVA P < 0.05, Table S3, Supporting information). Interestingly, *Janthinobacterium lividum*, which has been shown to have protective effects against the pathogen *B. dendrobatidis* (Harris *et al.* 2009), is also significantly associated with premetamorph *R. cascadae* at Section Line (N = 4) and present at 1.7% overall abundance (ANOVA P < 0.001, Table S3, Supporting information). OTUs that are significantly different between pre- and postmetamorphic stages of *R. cascadae* and the environment can be found in Table S3 (Supporting information).

Discussion

Host species effects on amphibian skin-associated bacterial communities

Sampling cohabiting amphibians at sites in close proximity allows us to tests the main factors structuring the amphibian skin microbiota, isolating the effect of species from the many confounding environmental factors at each site. We found that bacterial communities on amphibian skin were distinct among cohabiting species in the Central Valley sites, and that these

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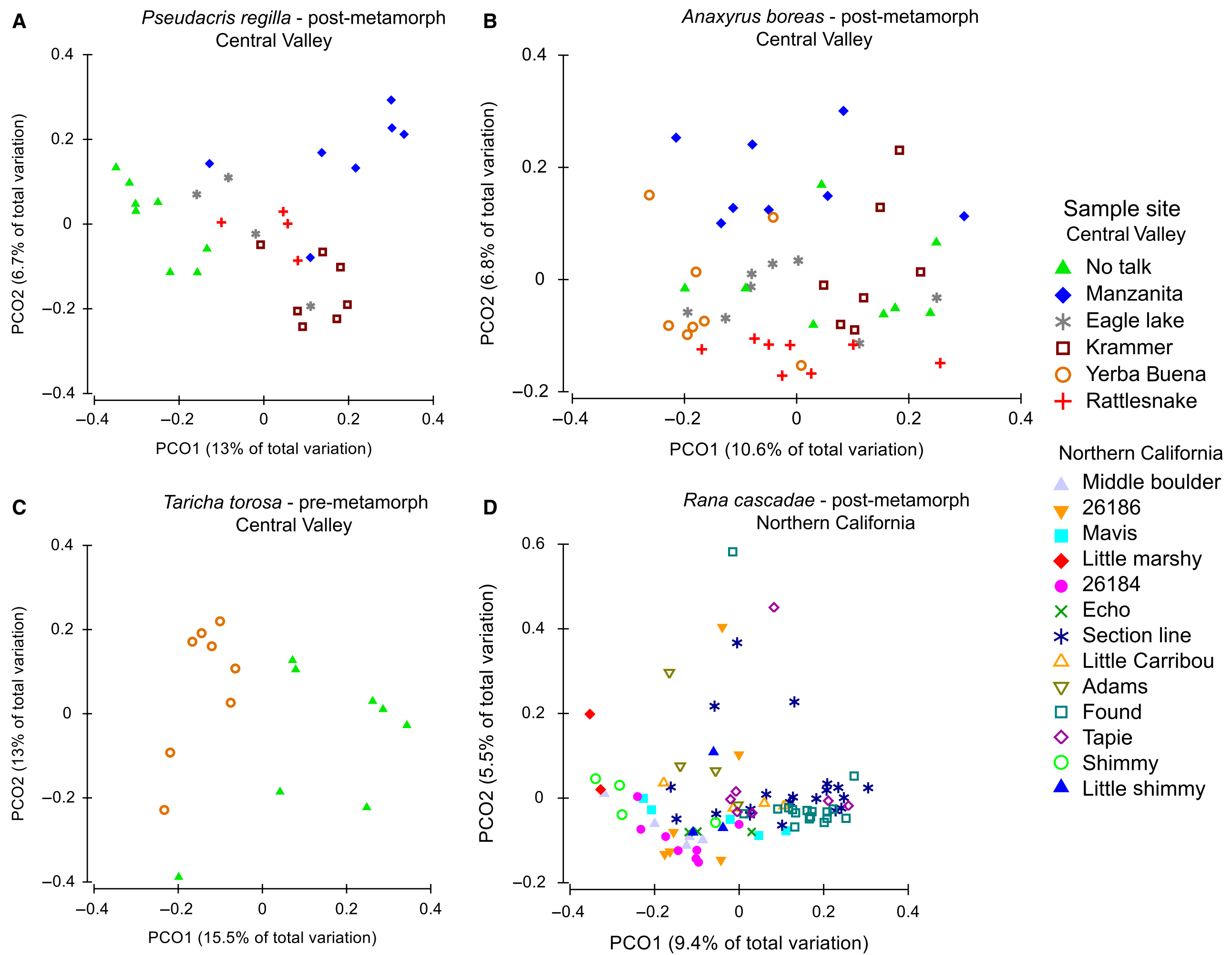

Fig. 5 Beta diversity of amphibian skin bacterial communities is structured by geographic locations within host species. (A) *P. regilla* subadults, (N = 30), (B) *A. boreas* subadults, (N = 47), (C) *T. torosa* larvae, (N = 16) and (D) *R. cascadae* adult, (N = 88), respectively. Diversity patterns were visualized using principle coordinates plots of unweighted UniFrac distances. Each point represents the skin bacterial community of an individual amphibian, symbol colour and shape indicate pond location.

differences were consistent across pond sites. In line with the findings of McKenzie *et al.* (2012), we observed that host species identity was the strongest predictor of bacterial communities on amphibian skin, and site explained additional variation. Thus, host differences probably drive assembly of the skin community. We were not able to explicitly examine the role of developmental stage in the Central Valley data set given the lack of different developmental stages present within each species (see Table 1). However, it does not appear that developmental stage is driving the differences we see between species because all species are significantly different from each other regardless of developmental stage (Fig. 3). Additionally, McKenzie *et al.* (2012) found similar separation by species in a data set that contained only larval stages from cohabiting amphibians in Colorado, indicating that even when developmental stages are better controlled for, host species emerges as the strongest predictor of skin-associated bacteria. In this California data set, each species appears to have unique dominant taxa (Fig. 4) and differing levels of diversity (Fig. 2). The skin of different amphibians and different life history stages may differ in physiological chemistry. Skin pH, defence peptides and organic molecules have only been studied in a few species (Conlon 2011; Rollins-Smith & Woodhams 2012). Better characterization of amphibian skin chemistry may be required to understand the drivers of skin microbial patterns. In other hosts, growing evidence suggests that many phenotypic attributes once thought to be the sole product of host genetics are influenced by host–microbial associations, including host metabolism, behaviour, mate choice and immune responses (Bravo *et al.* 2011; Neufeld *et al.* 2011; Sharon *et al.* 2011; Chung *et al.*

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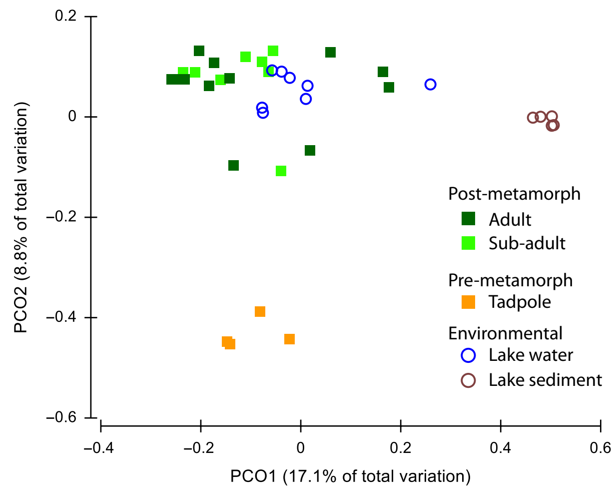

Fig. 6 Bacterial communities associated with amphibian skin vary according to life cycle stage within *R. cascadae*. Adult (N = 12), subadult (N = 8), tadpoles (N = 4), lake water (N = 8) sediment (N = 5). All amphibians were sampled from Section Line Lake in the Klamath region of Northern California and are compared with water and sediment from Section Line Lake. Diversity patterns were visualized principal coordinates plots of unweighted UniFrac distances.

2012). Given our results, we hypothesize that bacterial community structure of the amphibian skin is, in part, modulated by the host and may provide specialized services such as protection from environmental pathogens. Under certain scenarios such as tolerance to particular pathogens, the holobiont, or host and microbiota combined, may be considered the unit of selection (Rosenberg & Zilber-Rosenberg 2011).

Geographic site effects on amphibian skin-associated microbial communities

Pond site was a secondary factor that significantly influenced the skin bacterial communities. These results suggest that amphibian microbial communities may be selected from the environment by the amphibian host and thus may in fact be spatially associated with the host. Our results show that water quality attributes explain small amounts of the variation, indicating that additional nuances of environmental factors may be important but are not captured and explained in the present study. Single time point measurements of abiotic water quality may not have been sufficient to capture abiotic environmental signals that would correlate with the skin community variation across sites, especially if these signals are transient or integrated over time. It is also possible that host genetic variation across sites could contribute to site variation. In our study system, the sites were relatively close together (e.g. within the Joseph Grant Park in the Central Valley), and within the dispersal range abilities of these amphibians, so we do not expect that genetic variation played a large role in this data set but it could for others where host genetic variation is sharply delineated.

Host developmental effects on amphibian skin-associated microbial communities

Our results show that the skin-associated bacterial community of *R. cascadae* tadpoles is restructured following metamorphosis, concurrent with known changes in amphibian immune function (Rollins-Smith *et al.* 2011). Substantial structural and immunological changes occur in the skin during metamorphosis (Robinson & Heintzelman 1987; Faszewski & Kaltenbach 1995; Rollins-Smith 1998, Faszewski *et al.* 2008). Thus, developmental shifts in immunity and skin structure are two factors that may explain the observed shift in skin-associated microbiota. Major disturbances are often associated with greater risk of pathogen introduction and establishment (Shade *et al.* 2012). This is observed in amphibians where the transition from larva to metamorph is often accompanied by high mortality, with severe instances of chytridiomycosis immediately following metamorphosis in some species (*e.g.* Bosch *et al.* 2001). Tadpoles of *R. cascadae* had the lowest alpha diversity in this study, and their communities were dominated by OTUs from the genus *Pseudomonas* (Fig. 4). We know from a wealth of culture-dependent work, including cultivation of amphibian-associated microbes, that many Pseudomonads grow quickly and produce antimicrobials that can inhibit bacteria and fungi including *Bd* (Lauer *et al.* 2007; Woodhams *et al.* 2007). Even with low skin community diversity, tadpoles may be able to defend against pathogen establishment and maintain homeostasis through association and cultivation of pathogen-inhibiting microbes. However, the mechanism by which tadpoles may selectively cultivate Pseudomonads is not known. Mechanisms could include production of antimicrobial peptides, oligosaccharides in their mucus, mucosal antibodies and modulating mucosal adhesion (Rollins-Smith & Woodhams 2012). Low diversity was also seen for tadpoles of *L. catesbeianus*, but small sample numbers of adults prevented a direct comparison.

Due to our sampling methods, the use of a nondestructive sterile swabbing technique, we explored the amphibian skin community at only one depth, the mucus and potentially only the top layer of the amphibian skin. Thus, we may not capture all members of the community that specialize in deeper layers of skin tissue, such as granular or mucus glands (if microbes occur there). Studies that have attempted to explore various layers of human skin tissue find significant overlap between tissue layers, although not all OTUs found in

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the skin were associated with the superficial layers (Grice *et al.* 2008). Additionally, it is possible that not all of the bacteria obtained from the swab are actual skin symbionts of the amphibians. Nevertheless, as we found that amphibian species inhabiting the same pond harboured distinct bacterial communities, a significant proportion of the sequences were host-associated and not simply transient microbes from the pond environment.

Although host species/developmental stage was the main factor affecting the amphibian skin microbiota, the environment as a whole also had a statistically significant effect. We also observed substantial microbial shifts during development of *R. cascadae* occurring from the tadpole stage to the postmetamorphic stages. Thus host identity, site and developmental stage attributes each contribute to the symbiont community found on amphibians. Future 'common garden' experiments may be influential in untangling the genetic, biotic and abiotic factors that contribute and shape the amphibian skin microbiota.

We now know host microbiota can affect host phenotype and may partially explain variability in disease susceptibility observed across hosts, as susceptibility may be, in part, microbially mediated (Harris *et al.* 2009; Bletz *et al.* 2013). Studies of survivor populations hosting microbes known to exhibit and produce antipathogen compounds support the concept that immunity provided by microbiota can function as an extended phenotype (Woodhams *et al.* 2007; Rollins-Smith & Woodhams 2012). Ongoing work is linking amphibian immune function, beneficial microbial symbionts of the host and the promotion of holobiont health with microbial therapy (Bletz *et al.* 2013). Disentangling the contributive roles of the host and the environment is key to understanding the process of microbial colonization and assembly in and on hosts and improving these microbial therapies.

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Data accessibility

This study is deposited in the QIIME database with MiMARKS compliant (http://gensc.org/gc_wiki/index.php/MIMARKS) metadata (study #1620; http://www.microbio.me/qiime). All sequences and metadata from 195 amphibians and 32 environmental samples are also deposited in the European Bioinformatics Institute (accession ERP003541, www.ebi.ac@uk).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 QIIME output of OTUs significantly different between four amphibian species sampled at Joseph Grant. These OTUs were identified by running 'OTU Category Significance' in QIIME, which tests whether any of the OTUs in the output are significantly associated with a host species, using an ANOVA with FDR corrected *P*-values to determine significance. Only OTUs that comprised at least 0.1% of a species community are shown.

Table S2 (a) shows the number and percent of shared OTUs between amphibian species, soil and lake water, sampled at Joseph Grant, using the whole OTU table. (b) shows the number and percent shared OTUs between amphibian species, soil and lake water sampled at Joseph Grant after removing all rare OTUs (sequences with less then .0005% abundance).

Table S3 QIIME output of OTUs significantly different between developmental stages of *Rana cascadae* (pre versus post metamorphosis) and environmental samples taken at Section Line. These OTUs were identified by running 'OTU Category Significance' in QIIME, which tests whether any of the OTUs in the output are significantly associated with a developmental stage, using an ANOVA with FDR corrected p-values to determine significance. Only OTUs that comprised at least 0.1% of a species community are shown.

Table S4 Full OTU table and assigned taxonomy of all samples used in the analyses, listed by sample ID.

Table S5 Mapping file contains the metadata that accompany the sample ID found in the OTU table (Table S4, Supporting information).

Fig. S1 Bar graphs of relative abundance of sequences assigned to major bacterial taxonomic groups for each individual amphibian and environmental sample taken from Joseph Grant. These individual samples also provide the basis for Figure 3b.

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