The ISME Journal (2016) 10, 1682–1695

www.nature.com/ismej

ORIGINAL ARTICLE

Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*

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Symbiotic bacteria on amphibian skin can inhibit growth of the fungus *Batrachochytrium dendrobatidis* (*Bd*) that has caused dramatic population declines and extinctions of amphibians in the Neotropics. It remains unclear how the amphibians' skin microbiota is influenced by environmental bacterial reservoirs, host-associated factors such as susceptibility to pathogens, and pathogen presence in tropical amphibians. We sampled skin bacteria from five co-occurring frog species that differ in *Bd* susceptibility at one *Bd*-naive site, and sampled one of the non-susceptible species from *Bd*-endemic and *Bd*-naive sites in Panama. We hypothesized that skin bacterial communities (1) would be distinct from the surrounding environment regardless of the host habitat, (2) would differ between *Bd* susceptible and non-susceptible species and (3) would differ on hosts in *Bd*-naive and *Bd*-endemic sites. We found that skin bacterial communities were enriched in bacterial taxa that had low relative abundances in the environment. Non-susceptible species had very similar skin bacterial communities that were enriched in particular taxa such as the genera *Pseudomonas* and *Acinetobacter*. Bacterial communities of *Craugastor fitzingeri* in *Bd*-endemic sites were less diverse than in the naive site, and differences in community structure across sites were explained by changes in relative abundance of specific bacterial taxa. Our results indicate that skin microbial structure was associated with host susceptibility to *Bd* and might be associated to the history of *Bd* presence at different sites.

The ISME Journal (2016) **10,** 1682–1695; doi:10.1038/ismej.2015.234; published online 8 January 2016

Introduction

Symbiotic relationships between multicellular hosts and bacteria vary from species-specific obligate mutualisms (Nyholm and McFall-Ngai, 2004; Dale and Moran, 2006; Feldhaar, 2011) to beneficial relationships of macroorganisms with complex bacterial communities (Lindow and Brandl, 2003; Turnbaugh *et al.*, 2007; Knief *et al.*, 2012; Otani *et al.*, 2014). It is becoming clear that in many systems (e.g. plants, *Hydra*, corals, amphibians and humans) bacterial symbiotic communities play an important role in protecting hosts from pathogens (Rosenberg *et al.*, 2007; Harris *et al.*, 2009; Innerebner *et al.*, 2011; Khosravi and Mazmanian, 2013; Fraune *et al.*, 2014). Conversely, the diversity and structure of symbiotic bacterial communities can be altered following pathogen infection (Round and Mazmanian, 2009; Cárdenas *et al.*, 2012; Fierer *et al.*, 2012; Jani and Briggs, 2014).

In amphibians, some bacterial species in the mucous layer of the skin prevent *in vitro* growth of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Harris *et al.*, 2006; Brucker *et al.*, 2008a,b; Becker *et al.*, 2015a; Woodhams *et al.*, 2015). Moreover, bacterial additions to some amphibian hosts from North America can prevent or ameliorate the effects of chytridiomycosis, the disease caused by *Bd* (Harris *et al.*, 2009; Becker and Harris, 2010). Chytridiomycosis has caused dramatic population declines and extinctions of amphibian species all over the world (Berger *et al.*, 1998; Lips *et al.*, 2006). The Neotropics, in particular, have suffered dramatic population collapses in recent decades, but interestingly not all species declined, and susceptibility to *Bd* seems to vary among amphibian species (Lips *et al.*, 2006; Crawford *et al.*, 2010). For example, both terrestrial and arboreal species like *Craugastor fitzingeri* and *Espadarana prosoblepon,* respectively,

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Received 4 June 2015; revised 29 October 2015; accepted 6 November 2015; published online 8 January 2016

have persisted in highland and lowland tropical environments (Puschendorf *et al*., 2006; Crawford *et al*., 2010). In contrast, many species of the genus *Atelopus* have suffered dramatic population declines and extinctions across Central and South America (La Marca *et al*., 2005; Lips *et al*., 2006; Crawford *et al*., 2010). While there is differential susceptibility to *Bd* among amphibians (Brem and Lips, 2008; Crawford *et al*., 2010), the mechanisms driving that variation are not yet understood. However, given the role of symbiotic communities in disease resistance, different microbial community structures on skins might contribute to that variation. Unfortunately, there are little data available on the skin microbiota of wild susceptible and non-susceptible amphibians in the Neotropics (Flechas *et al*., 2012; Becker *et al*., 2014).

In addition, little is known about the biotic factors that shape amphibian skin microbial communities in different host species. Skin microbes may be influenced by host-associated traits, such as the chemical content of the skin mucus, including antimicrobial peptides, alkaloids, mucopolysaccharides and glycoproteins (Wells, 2007; Rollins-Smith, 2009; Conlon, 2011), as well as additional traits such as host behavior, skin shedding rate and diet (Meyer *et al*., 2012; Antwis *et al*., 2014). In North American amphibians, bacterial communities can vary among host species, populations within species and through development (McKenzie *et al*., 2012; Kueneman *et al*., 2014). Microbial communities in the environment can also influence the structure of the skin microbiota and could contribute to variation among host species. For example, microbial communities from the surrounding environment can serve as reservoirs and source pools of colonizers (Loudon *et al*., 2014; Kueneman *et al*., 2014; Walke *et al*., 2014). In two terrestrial salamander species, the relative abundances of cutaneous bacterial taxa were directly correlated with their relative abundances in the environment (Fitzpatrick and Allison, 2014; Loudon *et al*., 2014). However, skin bacteria from aquatic amphibians were enriched in taxa that occurred in low abundances in the environment (Kueneman, *et al*., 2014; Walke *et al*., 2014). Additional studies on co-occurring amphibian species with different habitats will be required to determine whether the skin microbiota is mainly constituted by unique residents or by bacterial species derived from the host's habitat.

It remains especially unclear how the amphibians' skin microbiota is influenced by environmental reservoirs, host-associated factors and pathogen presence in tropical amphibians since fewer studies have been conducted in tropical systems. Therefore, we conducted a field survey of amphibian species in Panama that vary in susceptibility to *Bd* and a more in-depth survey of one species across *Bd*-naive and *Bd*-endemic sites. We tested three primary hypotheses: (1) skin bacterial community structure differs from the surrounding environment regardless of the microhabitat that each species occupies in one location, (2) frog species in the same location have distinct cutaneous bacterial communities, and bacterial community structure is associated with variation in host susceptibility and (3) bacterial communities from a host species persisting in the presence of *Bd* differ from skin communities of the same host species at *Bd*-naive sites. To address the first two hypotheses we studied the skin microbiota of five frog species (*Atelopus certus, Colosthetus panamansis, E. prosoblepon, C. fitzingeri and Strabomantis bufoniformis*) from a *Bd*-naive site in the Darien province in Panama. These co-occurring species differ in their microhabitat and in their susceptibility to *Bd* based on previous field surveys and experiments (La Marca *et al*., 2005; Puschendorf *et al*., 2006; Woodhams et al., 2006; Brem and Lips, 2008; Crawford *et al*., 2010; Küng *et al*., 2014). First, we describe the relationship between skin and environmental bacterial communities associated with each host and explore differences among arboreal, riparian and terrestrial species in the absence of potential effects of *Bd* on the skin bacterial communities. Second, we compare bacterial communities of *Bd* susceptible and non-susceptible species, since susceptible species still occurred in high abundances at the study site. To address the third hypothesis, we compared the skin bacterial communities of one widely distributed species, *C. fitzingeri* at the *Bd*-naive site in the Darien province and at three *Bd*-endemic sites in the Panama and Colon provinces (Woodhams *et al*., 2008; Rebollar *et al*., 2014). As part of evaluating the third hypothesis, we determined whether site-level differences in the skin microbiota, if present, were associated with differences in environmental communities.

Materials and methods

Sample collection

To address our first two hypotheses, we sampled five frog species (Table 1) from 2–9 August 2012 that co-occur at Serrania del Sapo (Darien province, Panama), which is a *Bd*-naïve site according to quantitative PCR estimates obtained in this study (see results in Molecular methods and sequencing). In addition, the amphibian community observed in this site included a high abundance of susceptible species that do not occur anymore in *Bd*-infected sites (Hughey MC and Ibáñez R, unpublished data). Frogs were captured and skin swabs were collected according to previously published procedures (Rebollar *et al*., 2014). In addition to frog swabs, we collected 'perch swabs' from the exact place where each frog was found immediately after capturing an individual by swabbing the perch site for 20 s. We consider that perch samples represent an accurate collection of the microhabitat that each species occupies. However, it is important to mention that

Table 1 Host species, site characteristics and sample sizes of five frog species from different habitats in the *Bd*-naive site Serrania del Sapo

Host species (sample size)	*Family*	*Habitat*	*Associated environmental sample (sample size)*	*Susceptibility to* Bd *(references)*
Atelopus certus (8)	*Bufonidae*	Riparian	Rocks, palm, tree-trunk, soil (8)	Highly susceptible (La Marca *et al.*, 2005; Crawford *et al.*, 2010)
Craugastor fitzingeri (11)	*Craugastoridae*	Terrestrial	Leaf litter, logs, and soil (11)	Not susceptible (Puschendorf *et al.*, 2006; Crawford *et al.*, 2010)
Colosthetus panamansis (4)	*Dendrobatidae*	Riparian	Leaf litter and rock (4)	Ambiguous susceptibility (Woodhams *et al.*, 2008; Brem and Lips, 2008; Crawford *et al.*, 2010; Küng *et al.*, 2014)
Espadarana prosoblepon (7)	*Centrolenidae*	Arboreal	Tree leaves and branches (7)	Not susceptible (Woodhams *et al.*, 2006; Crawford *et al.*, 2010)
Strabomantis bufoniformis (10)	*Craugastoridae*	Riparian	Soil, logs and rocks (10)	Highly susceptible (Woodhams *et al.*, 2008; Crawford *et al.*, 2010)

Table 2 Field survey of *C. fitzingeri* across three *Bd*-endemic sites and one *Bd*-naive site in Panama

Host species (sample size)	*Location (province)*	*Elevation (m)*	*Environmental samples (sample size)*	Bd *status of each site (references)*
Craugastor fitzingeri (11)	Serrania del Sapo (Darien)	250	Leaf litter (4) Soil (2)	*Bd* naive (This study)
Craugastor fitzingeri (7)	Mamoni (Panama)	250	Leaf litter (3) Soil (3)	*Bd* endemic (Rebollar *et al.*, 2014)
Craugastor fitzingeri (15)	Soberania (Panama)	30	Leaf litter (3) Soil (3)	*Bd* endemic (Woodhams *et al.*, 2008; Rebollar *et al.*, 2014)
Craugastor fitzingeri (15)	Gamboa (Colon)	30	Leaf litter (3) Soil (3)	*Bd* endemic (Rebollar *et al.*, 2014)

perch samples might contain some bacterial operational taxonomic units (OTUs) that were rubbed off the frog skin. These sites included leaves, branches, logs, rocks, leaf litter or soil depending on where the individual was perched (Table 1). Host and perch swabs (total $N = 80$) were placed in 1.5 ml sterile microcentrifuge tubes and stored in liquid nitrogen during fieldwork. Once in the lab, tubes were kept at −80 °C until processing. Frog and perch sample sizes, amphibian species' habitats and susceptibilities to *Bd* are summarized in Table 1 and Supplementary Information.

To address our third hypothesis, *C. fitzingeri* were collected and swabbed from three additional lowland forests sites ($N = 37$) that are considered endemic for *Bd* (Soberania, Gamboa and Mamoni) in Colon and Panama provinces, Panama (Table 2; sample collection described in Rebollar *et al.,* 2014). These sites were closer together in comparison to the *Bd*-naive site, which is located in the Darien province (Figure 3e). To evaluate whether differences in skin bacteria across sites were associated with differences in environmental communities, we collected triplicate soil and leaf litter swabs at each site (Table 2). Sampling locations were chosen haphazardly, but were in the vicinity of where frogs were found. Environmental samples were collected by swabbing soil or leaf litter according to Walke *et al.* (2014).

Molecular methods and sequencing

Whole genomic DNA was extracted from all swabs using the DNeasy Blood and Tissue kit (Qiagen, Valencia CA, USA) according to the manufacturer´s instructions including a pretreatment with lysozyme. DNA extracted from swabs was used to amplify the V4 region of the 16S rRNA gene using barcoded primers (F515/R806) and PCR conditions adapted from Caporaso et al. (2011). Amplicons were quantified using Quantifluor (Promega, Madison, WI, USA). Composite samples for sequencing were created by combining equimolar ratios of amplicons from the individual samples, followed by cleaning with the QIAquick PCR clean up kit (Qiagen). Barcoded composite PCR products were sent to the Dana Farber Cancer Institute's Molecular Biology Core Facilities (Boston, MA, USA) for MiSeq Illumina sequencing using a 250 bp paired-end strategy.

A portion of the DNA extracts was used for the detection of *Bd* using Taqman real-time PCR assay according to Rebollar *et al.* (2014). *Bd* prevalence and infection intensity of *C. fitzingeri* from Soberania, Gamboa and Mamoni were previously reported (Rebollar *et al.*, 2014), and the same methods were used in this study for all frog samples at Sapo. We found no indication that *Bd* was present in Sapo, as quantitative PCR estimates from 40 individuals (five species) were all negative for *Bd* (mean = 0, CI 95% = 6.330×10^{-18}–0.087).

Sequence data processing

The 250 bp paired-end reads were filtered and processed with the Quantitative Insights Into Microbial Ecology pipeline (Caporaso *et al.,* 2010a). Forward and reverse reads were assembled using join_paired_ends.py. Assembled sequences were de-multiplexed and filtered to retain high-quality reads using the following filtering parameters: no N characters were allowed in retained sequences, no errors in barcode sequence were allowed, a minimum of five consecutive base pairs were needed to include a read and a maximum of five consecutive low-quality base pairs were allowed before truncating a read.

De-multiplexed samples were analyzed in two groups according to the hypotheses proposed in this study: The 'Sapo' data set included the samples from the five frog species from Sapo and their associated perch samples (Table 1), and the 'Crafit' data set was composed of *C. fitzingeri* and environmental samples across all four locations (Table 2). De-multiplexed 'Sapo' and 'Crafit' data sets were deposited in the NCBI sequence read archive with the accession numbers SRP065158 and SRP065432, respectively. De-multiplexed and filtered sequences from each data set were clustered into OTUs at a sequence similarity threshold of 97% with the UCLUST method (Edgar, 2010). Sequences were matched against the Greengenes database (May 2013 release; McDonald *et al.*, 2012), and those that did not match the database were clustered as *de novo* OTUs at 97% sequence similarity. Taxonomy was assigned using the RDP classifier (Wang *et al.*, 2007) and the Greengenes database. Representative sequences were aligned to the Greengenes database with PyNAST (Caporaso *et al.*, 2010b), and an ML phylogenetic tree was constructed with FastTree 2 (Price *et al.*, 2010). The OTU table for each data set was filtered using a minimum cluster size of 0.001% of the total reads (Bokulich et al., 2013). The number of reads per frog sample ranged from 39 500 to 169 344 in the Sapo data set and 12 335–148 210 in the Crafit data set. Both OTU tables were rarefied according to the sample with the lowest number of reads. The final rarefied OTU table for Sapo had 8006 OTUs and for Crafit had 6429 OTUs.

Data analysis

To address the relationship between host skin bacteria and environmental bacteria using the Sapo data set, we determined the proportion of shared and unique OTUs between host species and their respective perches. Shannon and Faith's Phylogenetic Diversity (PD) were calculated for both host and perch samples. *T*-tests were used to compare alpha diversity values between each host species and their perch samples. Bray Curtis, Weighted Unifrac and Unweighted Unifrac distance matrices were used to calculate the beta diversity and were visualized with a principal coordinates analysis. Differences in beta diversity between frog and perch samples were tested with non-parametric analysis of variance based on 999 permutations (PERMANOVA) using the software PRIMER-E (Clarke and Gorley, 2006). Kendal's tau ranked correlations of OTU relative abundance were calculated between each host sample and its respective perch sample (Fitzpatrick and Allison, 2014). These correlations were performed using (1) all OTUs and (2) abundant OTUs, which was defined as a relative abundance of 0.1% or higher as calculated by adding relative abundance values of host and perch samples.

To determine if skin bacterial communities differed among host species from the Sapo data set, we determined the proportion of shared and unique OTUs among host species. Shannon and PD were calculated, and ANOVA and *post hoc* Tukey's tests were carried out to determine differences between pairs of hosts. Beta diversity was calculated and visualized as above. PERMANOVAs were calculated as above to test for significant differences in beta diversity among host species. We used unweighted pair group method with arithmetic mean (UPGMA), a hierarchical clustering method based on the arithmetic mean, to determine clustering patterns across host species. UPGMA was used on Bray Curtis distances of mean OTU relative abundances at the genus level. Clusters obtained with UPGMA were evaluated with pairwise comparisons using analysis of similarities and Bonferroni adjusted *P*-values. UPGMA, Bray Curtis calculations and the resulting heatmap were completed using vegan package (Oksanen *et al.*, 2015) in R statistical package (R Core Team, 2014).

To address if bacterial community structure was associated to *Bd* presence, we compared the bacterial community structure of *C. fitzingeri* at three *Bd* endemic sites and one *Bd*-naive site. Shannon and PD diversity were calculated across sites, and ANOVA and *post hoc* Tukey's tests were conducted. Beta diversity was calculated and visualized as above. We used PERMANOVA to test for differences in bacterial beta diversity of *C. fitzingeri* across sites, between *Bd*-endemic and *Bd*-naive sites, between frog and environmental samples, and among environmental samples from different sites. Analysis of multivariate homogeneity of group dispersions was calculated to determine whether skin communities from different sites had different dispersion values using *betadisper* in vegan (Oksanen *et al.*, 2015). To determine the bacterial taxa that most likely explained differences between sites, we used the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata *et al.*, 2011). Classes were defined as *Bd*-endemic or *Bd* naive, and subclasses were the four sites. Following the precedent of previous studies, OTUs with LDA scores >2.0 were considered informative (Segata *et al.*, 2011; Clemente *et al.*, 2015; Eren *et al.*, 2015). All alpha and beta diversity metrics, principal coordinates analyses, analysis of similarities and relative abundance comparisons at the genus level were obtained with quantitative insights into microbial ecology pipeline (Caporaso *et al.*, 2010a).

Results

Skin bacterial communities were enriched in bacterial taxa that had low abundances in the environment regardless of host habitat

Of the total number of OTUs present on each frog species, pooling OTUs from all individuals per species, a high percentage was shared with its respective perch samples: *A. certus* (93.91%: 5061 shared OTUs of 5389 total OTUs), *Espadarana prosoblepon* (71.69%: 2264 shared OTUs of 3158 total OTUs), *Colostethus panamansis* (92.89%: 3307 shared OTUs of 3560 total OTUs), *Craugastor fitzingeri* (93.95%: 5631 shared OTUs of 5993 total OTUs) *and S. bufoniformis* (91.54%: 6344 shared OTUs of 6930 total OTUs). This indicates that the majority of the OTUs present on the frog skin were also present in the environment, and only a small percentage was unique to each frog species (Figure 1a). Perch samples had a higher percentage of unique OTUs in comparison with the percentage of unique frog samples, with the exception of *S. bufoniformis* (Figure 1a).

We compared the alpha diversity (Shannon and PD) of each host individual sample with its corresponding perch sample (Figure 1b and Supplementary Figure S1). In particular, host communities had a lower Shannon diversity in comparison with perch communities *A. certus*: $t=-6.71$, df = 12.37, $P<0.001$; *E. prosoblepon:* $t=-6.47$, df = 7.77, $P<0.001$; *C. panamansis:* $t=-6.39$, df = 12.37, $P<0.001$; *C. fitzingeri:* $t=-3.74$, df = 15.06, $P=0.001$), with the exception of *S. bufoniformis*, which had similar and not significantly different diversity values with respect to its perch ($t=-0.9$, df = 16.51, $P=0.37$). Similar results were obtained for PD diversity (Supplementary Figure S1). When comparing beta diversity values of all hosts and perch assemblages together, we also found significant differences between host and perch groups (PERMANOVA Pseudo-$F_{(1,76)}=8.7484$, $P=0.001$) and very little overlap in the bacterial communities as shown by distinct clustering patterns in the principal coordinates analysis (Figure 1c).

The relative abundances of OTUs found on individual frogs and on their corresponding perches were significantly and positively correlated for each host species (Figure 1d), with *S. bufoniformis* having the highest correlation values (*A. certus:* $R_\tau=0.289$, $P<0.001$; *E. prosoblepon:* $R_\tau=0.365$, $P<0.001$; *C. panamansis:* $R_\tau=0.295$, $P<0.001$; *C. fitzingeri:* $R_\tau=0.267$, $P<0.001$; *S. bufoniformis:* $R_\tau=0.429$, $P<0.001$). Interestingly, when we calculated ranked correlations with the most relatively abundant OTUs (OTUs $>0.1\%$) all of the correlations between frog and perch samples became negative and all but one were significant (Figures 1d; A. *certus:* $R_\tau=-0.235$, $P<0.001$; *E. prosoblepon:* $R_\tau=-0.07$, $P=0.117$; *C. panamansis:* $R_\tau=-0.363$, $P<0.001$; *C. fitzingeri:* $R_\tau=-0.266$, $P<0.001$; *S. bufoniformis:* $R_\tau=-0.166$, $P<0.001$). Overall, our results indicate that there was an inverse correlation between the most abundant OTUs present on hosts and on their perches, such that host-associated bacterial communities were enriched with rare environmental OTUs. Likewise, perch samples were enriched in bacterial taxa that were not abundant in frog samples (Figure 2d).

Skin bacterial community structures differed among hosts and were associated with Bd susceptibility

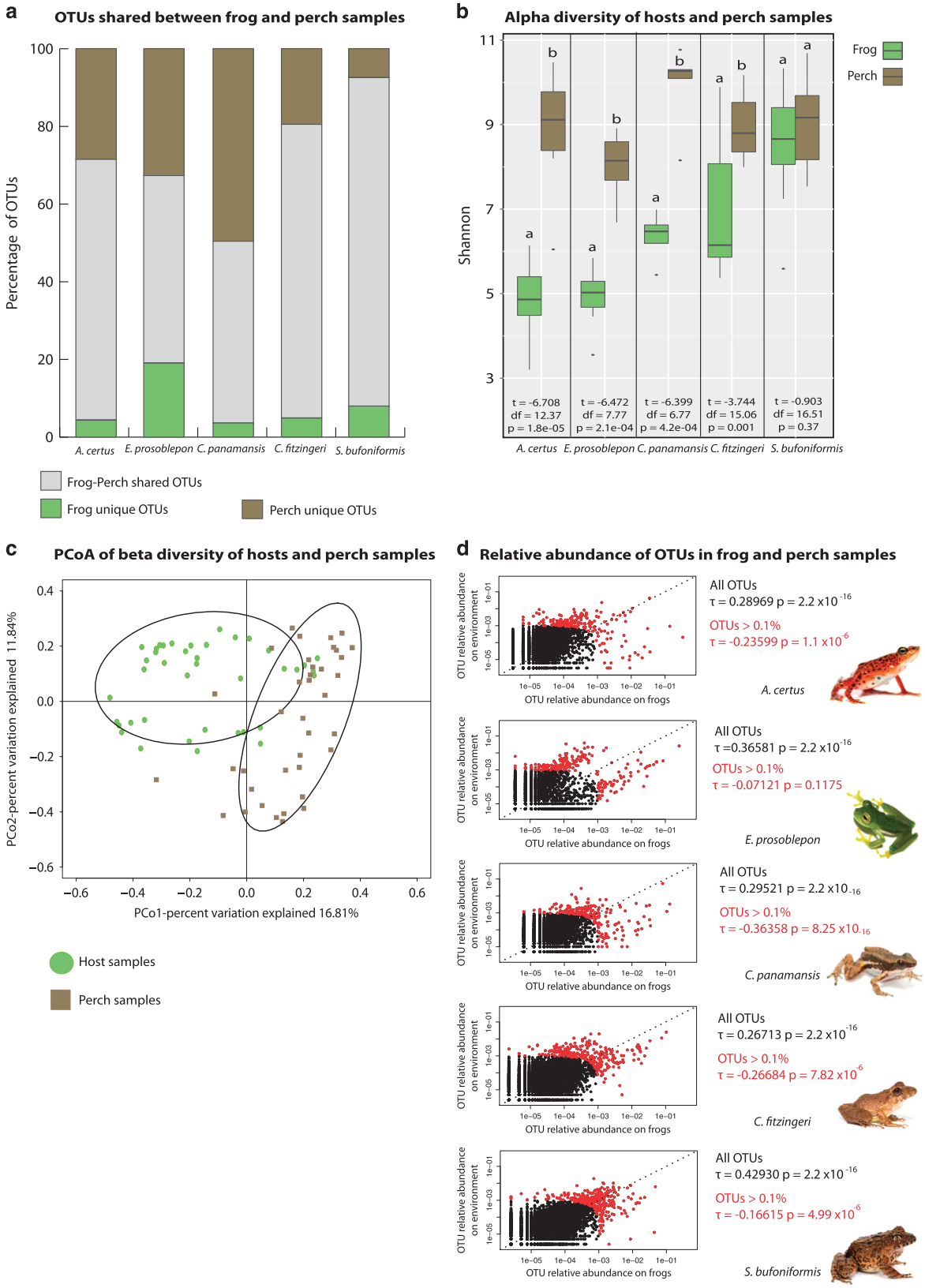
Of the total number of OTUs of all frog species together, there were only a few unique OTUs associated with each host species *A. certus* (0.24%), *E. prosoblepon* (0.37%), *C. panamansis* (0.37%), *C. fitzingeri* (2.36%) *and S. bufoniformis* (3.72%). These unique OTUs had extremely low relative abundances in comparison with OTUs that were shared among host species (Figure 2a). Moreover OTUs that were shared among the five host species accounted for the majority of the relative abundance on skin bacterial communities in all five species (Figure 2a).

Alpha diversity differed among host species, as assessed with Shannon index (ANOVA $F_{(4,35)}=14.42$, $P<0.001$; Figure 2). For example, *A. certus* and *E. prosoblepon* each had significantly lower values than *C. fitzingeri, whereas* diversity of *S. bufoniformis* was higher than all other species (Tukey's tests: *C. fitzingeri–A. certus,* $P=0.004$; *C. fitzingeri–E. prosoblepon*, $P=0.009$; *S. bufoniformis–A. certus*, $P<0.001$; *S. bufoniformis–E. prosoblepon*, $P<0.001$; *S. bufoniformis–C. panamansis*, $P=0.03$; *S. bufoniformis–C. fitzingeri*, $P=0.04$; Figure 2b). PD also differed among host species (ANOVA $F_{(4,35)}=6.511$, $P<0.001$). For example, the bacterial community diversity in *S. bufoniformis* was significantly higher from *E. prosoblepon* and *C. fitzingeri* (Supplementary Figure S2).

Beta diversity analyses of bacterial skin communities indicated that most of the differences among communities were due to differences in OTU relative abundance and not from differences in OTU presence/absence (Figure 2c): Host bacterial communities differed significantly using Bray Curtis (PERMANOVA Pseudo-$F_{(4,35)}=5.0152$, $P=0.001$; Figure 2) and Weighted Unifrac (PERMANOVA

Figure 1 Frog skin and perch bacterial communities of five frog species at Sapo (**a**) Percentage of shared and unique OTUs of each frog species and perch samples. (**b**) Alpha diversity (Shannon) of each host and its respective perch. *T*-test results are shown at the bottom of each paired comparison. (**c**) Beta diversity of hosts and perch samples. Principal coordinate analysis of Bray Curtis distances. Ellipses show confidence Intervals (CI) of 95% for each sample type. (**d**) Relative abundances of OTUs on each frog species and its respective perch samples. Red dots show OTUs with a total relative abundance $>0.1\%$. Kendall's ranked correlations, $R_{\tau au}$, and *P*-values are shown to the right of each plot. Photo credits: Brian Gratwicke (*A. certus, E. prosoblepon, C. panamansis, C. fitzingeri*) and Brad Wilson (*S. bufoniformis*).

Pseudo-$F_{(4,35)} = 7.5759$, $P = 0.001$), both of which consider the relative abundance of OTUs. In contrast, Unweighted Unifrac, which does not consider relative abundance of taxa showed no significant differences among host samples (PERMANOVA Pseudo-$F_{(4,35)} = 0.9912$, $P = 1$).



Bacterial community structure was associated with host species susceptibility to *Bd:* Hierarchical clustering of Bray Curtis distances using UPGMA revealed that the non-susceptible species (*E. prosoblepon*, *C. fitzingeri* and *C. panamansis*) had a similar community structure that contrasted with the community structures of the two highly susceptible species (*A. certus* and *S. bufoniformis*), that each formed a distinct cluster (Figure 2d). Skin bacterial communities of *A. certus* had a distinct composition of OTUs from the rest of the host and perch samples whereas *S. bufoniformis* clustered with all perch samples (Figure 2d). The clustering pattern among frogs was confirmed by pairwise analyses of similarities: no significant differences were found among *E. prosoblepon*, *C. fitzingeri* and *C. panamansis,* whereas *A. certus* and *S. bufoniformis* were each significantly different from all other host species (see statistical results in Supplementary Table S1).

In terms of the community structure, *E. prosoblepon*, *C. fitzingeri* and *C. panamansis* (non-susceptible species) had communities dominated by the genera *Pseudomonas* and *Acinetobacter* (both in Gammaproteobacteria). Skin bacterial communities on the *Bd*-susceptible riparian frog *A. certus* were dominated by OTUs from the genera *Pedobacter* and *Hylemonella* (Sphingobacteria and Betaproteobacteria, respectively). In contrast, the skin bacterial community of the *Bd*-susceptible species *S. bufoniformis* had a high abundance of OTUs from the families Verrucomicrobiaceae (Verrucomicrobia) and Comamonadaceae (Betaproteobacteria).

Site and pathogen presence, but not the community structure of environmental bacteria, influenced skin bacterial communities of C. fitzingeri

We evaluated differences in community structure of *C. fitzingeri* across sites with different *Bd* histories. The three *Bd*-endemic sites (Gamboa, Soberania and Mamoni) were compared with Sapo, the *Bd*-naive site (Figures 3d and e). Significant differences in Shannon and PD alpha diversity were found across sites (ANOVA $F_{(9,68)} = 7.279$, $P < 0.001$ and $F_{(3,44)} = 6.136$, $P = 0.001$ respectively). For Shannon, differences were driven by bacterial communities from Sapo, which had a higher diversity in contrast to the rest of the sites (Tukey's tests: Sapo-Mamoni, $P = 0.002$; Sapo-Soberania, $P = 0.04$; Sapo-Gamboa, $P < 0.001$; Figure 3a). In the case of PD, Gamboa had significantly lower diversity values in comparison to Sapo and Soberania (Tukey's tests: Gamboa-Soberania, $P = 0.005$; Gamboa-Sapo, $P = 0.008$). All other pairwise comparisons for Shannon and PD were not significant ($P > 0.05$).

Beta diversity of *C. fitzingeri* skin communities based on Bray Curtis distances differed between *Bd*-endemic sites and the *Bd*-naive site (Figure 3b; PERMANOVA Pseudo-$F_{(1,46)} = 10.556$, $P = 0.001$) and across sites (Figure 3; PERMANOVA Pseudo-$F_{(3,44)} = 9.688$, $P = 0.001$). Dispersion values across sites were significantly different with Mamoni having the least variation of all sites, and Sapo and Gamboa having the highest variation (ANOVA $F_{(3,44)} = 4.5608$, $P = 0.007$; Supplementary Figure S3).

To evaluate if the differences found on *C. fitzingeri* across sites were associated with distinct environmental bacterial communities, we compared frogs' skin samples with samples taken from soil and leaf litter at all sites (Table 2). Significant differences were found among environmental samples based on site and sample type (soil or leaf litter) (Supplementary Figures S4a and b, PERMANOVA site: Pseudo-$F_{(3,20)} = 1.509$, $P = 0.003$ and sample type: Pseudo-$F_{(1,22)} = 2.727$, $P = 0.001$). Importantly, we found significant differences between frog and environmental samples that formed distinct clusters in the principal coordinates analysis (Supplementary Figure S4c; PERMANOVA Pseudo-$F_{(2,69)} = 10.416$, $P = 0.001$). Overall, frog microbial communities were more similar to each other than any of them were to the bacterial communities in their environments (Supplementary Figure S4c).

The mean relative abundance profiles of frog OTUs indicated that *C. fitzingeri* at Sapo had a different bacterial composition compared with the three *Bd*-endemic sites (Figure 3d). The *Bd*-endemic sites (Gamboa, Soberania and Mamoni) were dominated by OTUs from the genera *Pseudomonas* (Gammaproteobacteria), *Cellulomonas* and *Sanguibacter* (both Actinobacteria), whereas the *Bd*-naive site (Sapo) had a low proportion of these taxa. The *Bd*-naive site was dominated by OTUs from the genus *Acinetobacter* (Gammaproteobacteria). In addition, Gamboa had a greater proportion of OTUs from the family *Comamonadaceae* (Betaproteobacteria) (Figure 3). We used the LEfSe method (Segata *et al.*, 2011) to determine the bacterial OTUs that are most likely explaining the differences between the *Bd*-endemic sites and the *Bd*-naive site (Figure 4a). Of the 6429 OTUs identified in *C. fitzingeri,* 244 were identified that distinguish *Bd*-endemic and *Bd*-naive classes (LDA scores > 2) (Supplementary Table S2). Forty-seven of these OTUs had higher relative abundances in the *Bd*-endemic sites. These 47 OTUs spanned 2 phyla and 3 classes, with the orders Pseudomonadales and Actinomycetales accounting for the majority of the OTUs (45.5% and 43.2%, respectively; Figure 4b and Supplementary Table S2). In contrast, 197 OTUs where more abundant in the *Bd*-naive site, and these taxa were more diverse, spanning 8 phyla and 16 classes (Figure 4 and Supplementary Table S2).

Discussion

We hypothesized that the skin bacterial communities of amphibian hosts would be distinct from environmental samples. We found that the skin communities on the five species studied from Sapo shared the majority of their OTUs with their respective perch

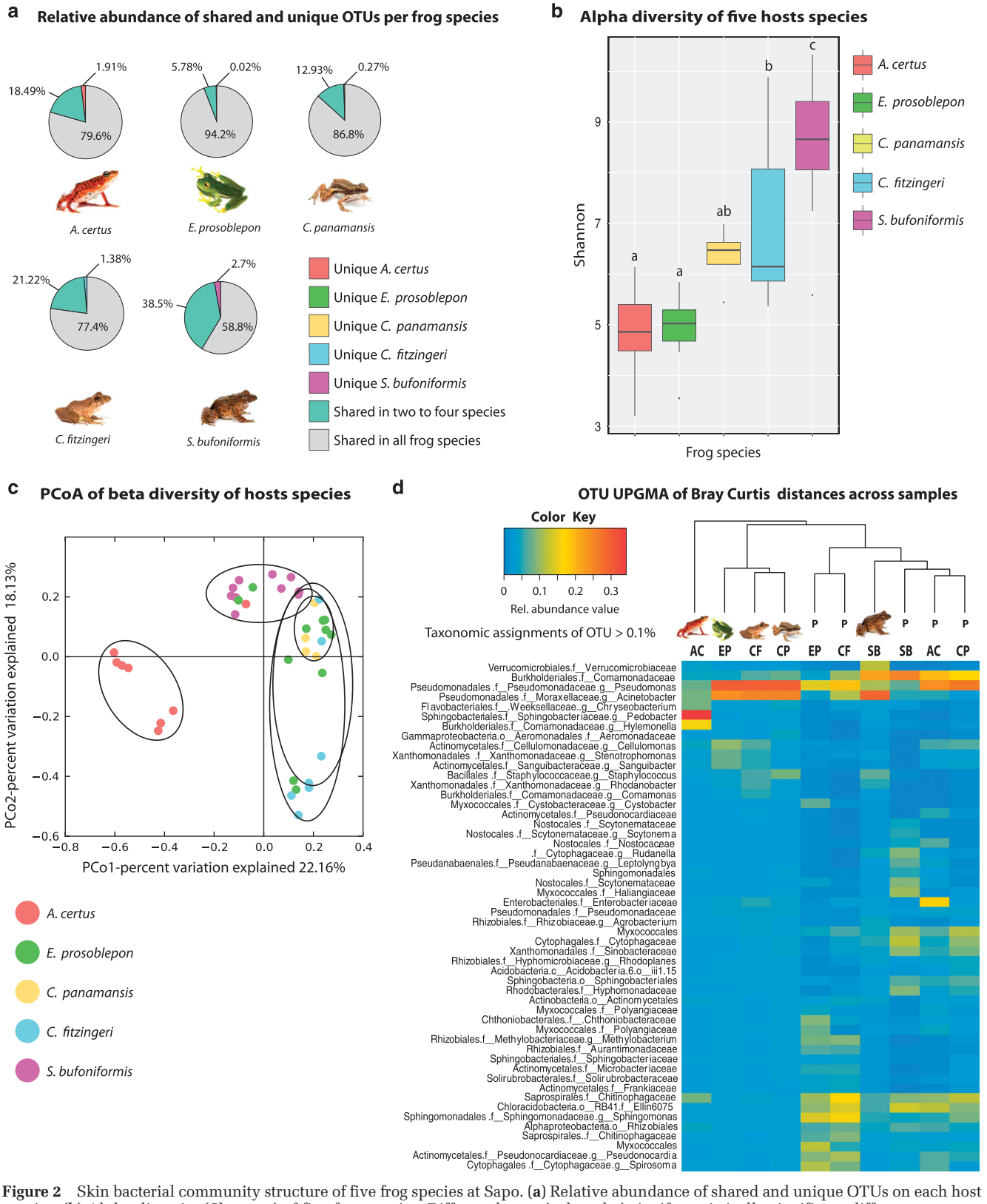


Figure 2 Skin bacterial community structure of five frog species at Sapo. (**a**) Relative abundance of shared and unique OTUs on each host species. (**b**) Alpha diversity (Shannon) of five frog species. Different letters (a, b and c) signify statistically significant differences among hosts, as indicated by the Tukey's *post hoc* tests. (**c**) Beta diversity of five host species. Principal coordinate analysis of Bray Curtis distances. Ellipses show confidence Intervals (CI) of 95% for each host species. (**d**) UPGMA and heatmap of bacterial genera with relative abundances >0.1% across five hosts species and their perch. Rows are bacterial genera. Columns are samples. Colors indicate taxa with a higher (red) or lower (blue) relative abundance in each sample. P, perch samples; AC, *A. certus*; EP, *E. prosoblepon*; CP, *C. panamansis*; CF, *C. fitzingeri*; SB, *S. bufoniformis*. Photo credits: Brian Gratwicke (*A. certus, E. prosoblepon, C. panamansis, C. fitzingeri*) and Brad Wilson (*S. bufoniformis*).

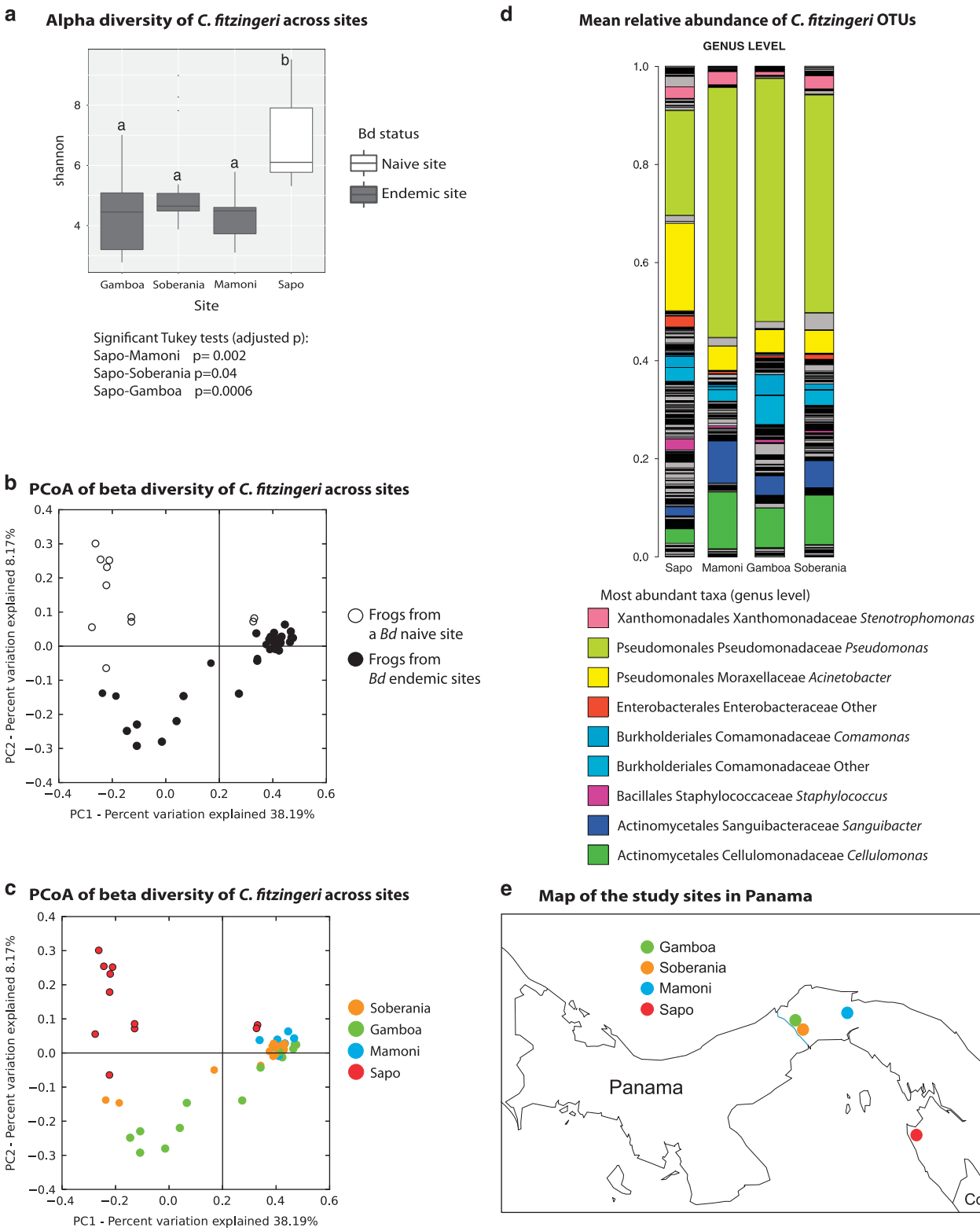


Figure 3 Skin bacterial community structure of *C. fitzingeri*. (**a**) Alpha diversity (Shannon) of *C. fitzingeri* across four regions (ANOVA $F_{(9,68)} = 7.279$, $P = 4.5 \times 10^{-4}$). Different letters (a and b) signify statistically significant differences among hosts, as indicated by the Tukey's *post hoc* tests. (**b** and **c**) Beta diversity of *C. fitzingeri* across four sites: Gamboa, Soberania, Mamoni and Sapo. Principal coordinate analyses are based on Bray Curtis distances. (**d**) Stacked bar chart of mean relative abundances of bacterial taxa (genus level) of *C. fitzingeri* across sites. Colored bars (legend) indicate the nine most abundant taxa. (**e**) Map of the four study sites in Panama.

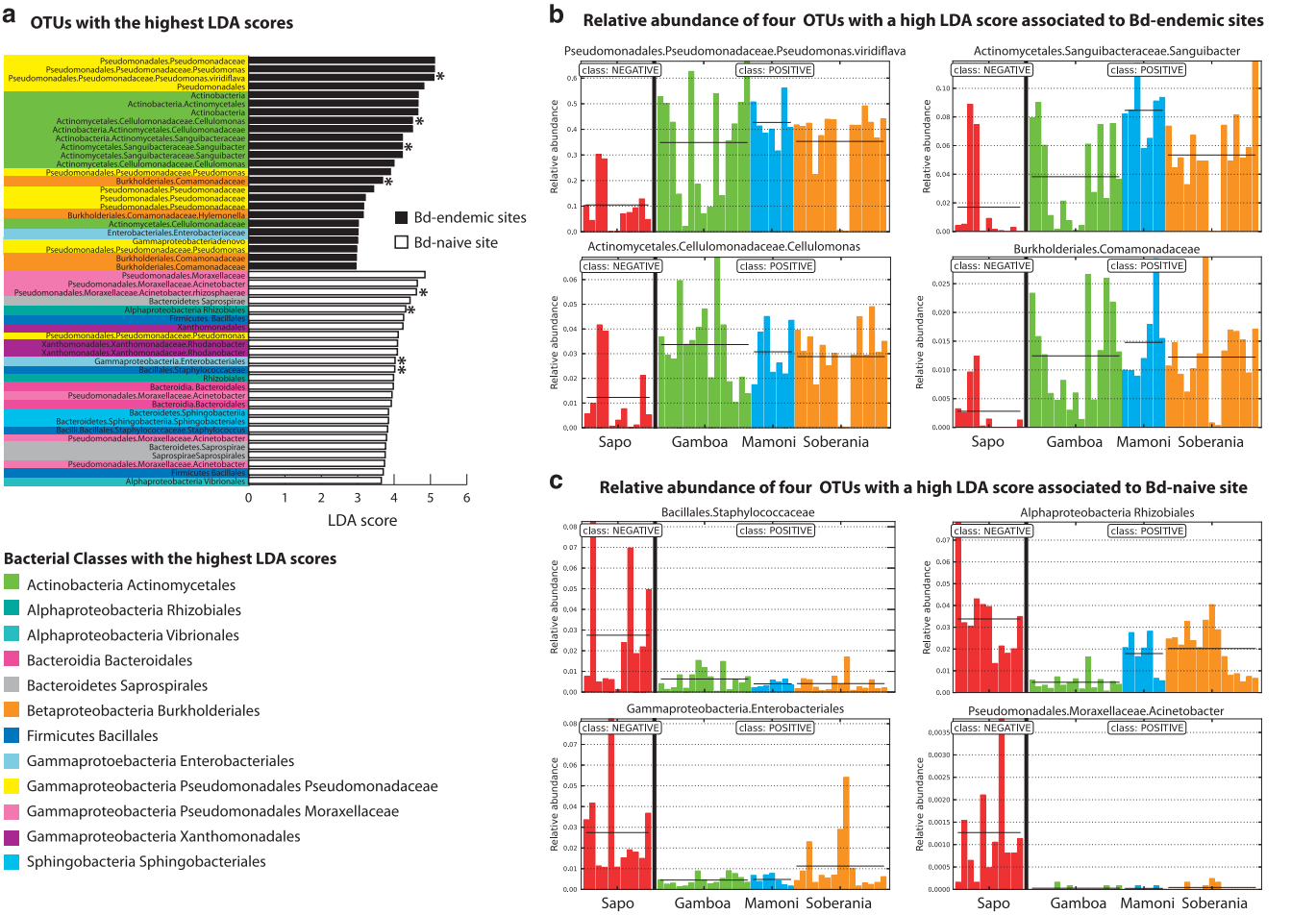


Figure 4 Bacterial OTUs that differ between *Bd*-endemic and *Bd*-naive sites based on LEFfSe analysis. (**a**) OTUs with the highest linear discriminant analysis (LDA) scores from *Bd*-endemic sites and the *Bd*-naive site. Bars on graph indicate LDA values. Asterisks indicate taxa that are shown in (**b**) and (**c**). (**b**) Four representative OTUs with high relative abundances in the *Bd*-endemic sites. (**c**) Four representative OTUs with high relative abundances in the *Bd*-naive site. OTUs chosen for (**b**) and (**c**) represent examples of OTUs with the following attributes: (1) high LDA scores, (2) they span different taxonomic groups and (3) OTUs that were identified in only one of the groups (infected or naive). Bars in (**b**) and (**c**) correspond to individual frogs and colors correspond to the four sites: Sapo, Gamboa, Mamoni and Soberania.

environments. However, the relative abundances of the most abundant microbes on the skin and in the environment were distinct. This trend was consistent among host species, regardless of the habitat type that each species occupied. Even though we consider perches to contain an accurate representation of the microbiota found in the microhabitat that each frog species occupies, it is possible that some frog OTUs might be rubbed off onto perches in the same way *Bd* is (Kolby *et al*., 2015). However, even considering this caveat, we found clear differences between frog and perch samples. As in a previous study of aquatic amphibian hosts (Walke *et al*., 2014), skin communities in this study were enriched in OTUs that had very low abundances in the environment, suggesting that the skin represents a unique niche that favors the growth of specific bacterial taxa. Skin mucus requires a stable pH for gas exchange, and it contains several biomolecules with protective roles against desiccation (Edwards, 1979; Boutilier *et al*., 1992; Wells, 2007) and pathogens (Conlon, 2011). Thus, the particular composition of the mucous layer likely influences the bacterial community structure and favors the growth and persistence of specific taxa even though the skin is constantly exposed to the outside environment. In the case of epithelial and gut communities in *Hydra* and mice, respectively, the production of specific antimicrobial peptides facilitated the colonization of particular bacterial species and contributed to the stability of microbial communities (Fraune *et al*., 2010; Salzman *et al*., 2010; Franzenburg *et al*., 2013).

We also hypothesized that at the *Bd*-naive site, Sapo, there would be differences in the skin microbiota between *Bd*-susceptible and non-susceptible host species. We found that the skin microbial communities clustered based on the host species' susceptibility to *Bd*. We identified clear similarities in the bacterial community structure of the three non-susceptible species *C. fitzingeri*, *E. prosoblepon* and *C. panamansis*. These three

species differ in natural history and habitat use, but they seem to be colonized by the same symbiotic bacterial taxa. The most abundant OTUs on the skins of *C. fitzingeri*, *E. prosoblepon* and *C. panamansis* were members of the genera *Pseudomonas* and *Acinetobacter*. Both genera are known for their functional diversity and ubiquity in different environments (Silby *et al.*, 2011), and the genus *Pseudomonas* are commonly found on other amphibian species at various developmental stages (Woodhams *et al.*, 2007; Flechas *et al.*, 2012; Kueneman *et al.*, 2014; Loudon *et al.*, 2014; Walke *et al.*, 2014). Some *Pseudomonas* species produce metabolites that inhibit the growth of *Bd in vitro* (Brucker *et al.*, 2008a; Becker *et al.*, 2015a), and some *Acinetobacter* strains isolated from amphibians inhibit *Bd in vitro* (Woodhams *et al.*, 2015). Moreover, both *Pseudomonas* and *Acinetobacter* species are able to form biofilms that provide structure and protection to bacterial communities in other biological systems (Drenkard and Ausubel, 2002; Wei and Ma, 2013; Longo *et al.*, 2014). Our results suggest that skin microbial structure can be a reflection of host susceptibility to *Bd* or can have a fundamental role in protecting hosts against *Bd.*

The highly susceptible species *A. certus* and *S. bufoniformis* had skin communities that were distinct from the non-susceptible species and from each other. Interestingly, the most abundant bacterial taxa (Sphingobacteriaceae and Comamonadaceae) of the highly susceptible species *Atelopus zeteki* were also the most abundant in *A. certus* (Becker *et al.*, 2014), which suggest that species that are phylogenetically related might have similar skin chemical conditions. Additional studies spanning a larger number of species will be needed to evaluate the effect of host phylogenetic relatedness on skin microbes. In contrast to *A. certus*, the skin of *S. bufoniformis* was enriched in different taxa and was more closely associated with environmental samples. Remarkably, the three species that share a common environment, *A. certus*, *S. bufoniformis* and *C. panamansis* had the most distinct community structure, which supports the idea that skin microbiota is more influenced by the host than by environmental microbes.

Our final hypothesis was that there would be variation in the skin microbiota of amphibian hosts found in *Bd*-endemic as compared with *Bd*-naive sites. *C. fitzingeri* is a widespread terrestrial species that has persisted in lowland and highland forests despite the presence of *Bd* (Puschendorf *et al.*, 2006; Crawford *et al.*, 2010). Our results indicated differences in bacterial community structure across populations of *C. fitzingeri* as seen in other amphibian species from North America (McKenzie *et al.*, 2012; Kueneman *et al.*, 2014; Walke *et al.*, 2014). In *Bd*-endemic sites we found clear enrichments in OTUs from the *Cellulomonas* and *Sanguibacter* genera, as well as from *Pseudomonas*. These former genera have been identified in other amphibian species (Becker *et al.*, 2014; Jani and Briggs, 2014; Walke *et al.*, 2014). Actinomycetales in general are known to produce antibiotics in natural environments (Waksman and Lechevalier, 1962). In addition, members of the genus *Cellulomonas* can degrade cellulose and chitin via extracellular enzyme production (Coughlan, 1985; Reguera and Leschine, 2001). As in the case of other chytrid fungi, *Bd* zoosporangia cell walls are made of chitin (Rasconi *et al.*, 2009; Friesen and Kuhn, 2012) and could therefore be a target for chitin-degrading bacteria (Kitamura *et al.*, 2002). Even though we found clear differences in bacterial community structure across sites with different *Bd* histories (infected and naive sites), a recent study showed that variation in community structure does not correlate with host infection status of *C. fitzingeri* and other species within these regions (Belden *et al.*, 2015). However, the latter study did not include *Bd*-naive areas.

In addition to the presence of *Bd*, skin bacterial communities on *C. fitzingeri* could differ across sites due to their interaction with different environmental communities. Indeed, skin bacterial communities were distinct across sites; however they were always more similar to each other than any of them were to the bacterial communities in their environments. This result confirms that cutaneous microbial communities are distinct from bacterial environmental communities and that location effects in *C. fitzingeri* skin communities were not directly driven by the structure of environmental communities. It is important to consider that *Bd*-infected sites were closer to each other (Colon and Panama Provinces) in contrast to the Sapo site at the Darien Province (Figure 3e). Thus, differences between infected and naive sites could be confounded by geography, which in turn may be associated with habitat differences. In this respect, abiotic factors such as temperature and humidity may differ among sites and could directly or indirectly influence skin microbial communities (Kueneman *et al.*, 2014). s another possibility, genetic differentiation between frog populations could explain differences in bacterial communities. Indeed, genetic divergence between Sapo and sites in the Colon and Panama provinces has been detected in *C. panamansis*, *S. bufoniformis* and *Hemiphractus fasciatus* (Crawford *et al.*, 2013). It is possible that *C. fitzingeri* may have diverged in a similar way. Thus, the differences in OTU relative abundance among sites could also be explained by differences in hosts' genetic divergence patterns.

Our results suggest that skin microbial structure is mostly influenced by host-associated traits and is less affected by environmental microbes. In addition, we can envision at least three possible scenarios to explain the effect of *Bd* on persisting amphibian populations: (1) *Bd* directly affects the skin microbiota and only certain bacterial groups will be selected in response to the infection (Jani and Briggs, 2014). In this context, non-susceptible hosts

might harbor *Bd*-inhibitory bacteria that are selected for in the presence of *Bd* and consequently these bacteria become enriched. (2) Changes in gene expression on the host occur in the presence of pathogens, such as genes coding for AMP production (Salzman *et al.*, 2010; Franzenburg *et al.*, 2013). This in turn modifies the skin microbial structure. (3) Natural selection acts on hosts with particular genetic profiles and their associated microbial communities, as suggested in a recent study on *A. zeteki* (Becker *et al.*, 2015b). Experimental studies with non-susceptible frog species will be required to test these three possible scenarios.

Overall, skin bacterial communities in tropical frogs had skin bacterial communities that were distinct from environmental bacterial communities, and this occurred regardless of the host habitat. The main distinctions between skin and environmental bacteria could be explained by differences in relative abundance of the most abundant OTUs. We found differences in the community structure of *Bd* susceptible and non-susceptible species, suggesting that skin symbiotic bacteria are either a cause or a reflection of the host susceptibility to *Bd*. Finally, skin microbial communities on *C. fitzingeri* differed between *Bd*-endemic and one *Bd*-naive site. The functions of skin symbiotic bacteria likely go beyond their role in pathogen protection, and therefore genetic and functional studies of skin bacteria are required to more fully understand the microbial–host interactions that occur in the amphibian skin microbiome.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We thank guides Gustavo, Alonzo and Evelio for assistance in the field. We thank M Bletz for technical assistance in the laboratory and S Ramirez-Barahona for constructive comments to the manuscript. Scientific collection permits were provided by the Panamanian authorities (Autoridad Nacional del Ambiente): permits SE/A-47-12, SEX/A-65-12, SEX/A-77-12 and SEX/A-89-12. Animal care protocols were approved by the Smithsonian Tropical Research Institute's Animal Care Committee: protocol 2011-1110-2014 and by Virginia Tech's Animal Care Committee: protocol 11-105- BIOL. This project was supported by the National Science Foundation Grants: DEB-1136602 (to RNH) and DEB-1136640 (LSB).

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