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Community richness of amphibian skin bacteria correlates with bioclimate at the global scale.

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1 **Community richness of amphibian skin bacteria correlates with bioclimate at the global**
2 **scale**
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52

54 **Abstract**

55

56 Animal-associated microbiomes are integral to host health, yet key biotic and abiotic factors that

57 shape host-associated microbial communities at the global scale remain poorly understood. We

58 investigated global patterns in amphibian skin bacterial communities, incorporating samples from

59 2,349 individuals representing 205 amphibian species across a broad biogeographic range. We

60 analyzed how biotic and abiotic factors correlate with skin microbial communities using multiple

61 statistical approaches. Global amphibian skin bacterial richness was consistently correlated with

62 temperature-associated factors. We found more diverse skin microbiomes in environments with

63 colder winters and less stable thermal conditions, compared to environments with warm winters

64 and less annual temperature variation. We used bioinformatically predicted bacterial growth

65 rates, dormancy genes, and antibiotic synthesis genes, as well as inferred bacterial thermal growth

66 optima to propose mechanistic hypotheses that may explain the observed patterns. We conclude

67 that temporal and spatial characteristics of the host's macro-environment mediate microbial

68 diversity.

Introduction

Microbial symbionts influence animal physiology, evolution, and health in a variety of ways [1], yet factors governing global-scale patterns in diversity of host-associated microbes are not fully understood. The largest scale study to date found that the primary predictor of microbial diversity was whether the sample was host-associated versus free-living. Furthermore, for host-associated communities, animal versus plant hosts and gut versus skin were the strongest predictors of microbial communities[1]. This underscores the importance of host-association in shaping a unique subset of the earth's microbes; however, most host microbiome studies are from geographically- and taxonomically-focused studies. A predictive framework for these communities at the global scale is lacking, but vital for understanding ecology and evolution of host-associated microbiomes [2], detection of dysbiosis, or elucidating changes in microbiome functions [3].

Generalizable rules for predicting free-living microbial taxa are increasingly being explored. For instance, studies have found that geographic ranges of environmental bacteria decrease towards the equator following Rapoport's Rule [4,5]. In addition, composition and diversity of environmental microbiomes vary with latitude [6–10], and are known to be structured by abiotic factors, such as salinity, pH, temperature, oxygen and nutrients [11–15]. Thus, while consistent patterns have been detected for environmental microbes, it is uncertain if generalizable rules govern composition of naturally-occurring host-associated microbiomes. In modern humans, diet and lifestyle are important drivers of gut community similarity [16], and for mammals broadly, diet and host phylogeny are strong predictors of the gut microbiome [17,18].

Amphibian skin is a leading model system to explore host-associated microbial community structure. The skin can be sampled non-destructively, and the need to understand skin microbial ecology is hastened by emerging pandemic diseases [3,19,20]. In recent decades, amphibian species have been decimated by the invasive fungal pathogens *Batrachochytrium dendrobatidis* [21,22], and more recently, *B. salamandrivorans* [23–25]. Previous research on amphibians from aquatic systems has found that amphibian host identity is the strongest predictor of skin-associated bacteria, while developmental life stage and environment are secondary predictors [26–28]. Other studies have found host microhabitat preferences and ecological factors best predict amphibian skin microbiomes [29,30]. At a local scale, amphibian skin microbial diversity varies temporally [31–33] and is reduced when hosts are exposed to habitat destruction, microclimate shifts, and captivity [34–36]. Indeed, the research community has generated substantial knowledge on microbial communities

3

101 of particular amphibians [3], but typically has focused on small geographic areas, leaving out

102 analysis of climatic variables. While these individual advancements are valuable, large global-

103 scale datasets are needed to evaluate how environmental vs. intrinsic factors mediate composition

104 and diversity of amphibian-associated microbiomes. Undeniably, across all investigations into

105 host-associated microbiomes, abiotic effects at the local scale are typically weak, although often

106 statistically detectable, and the influence of climatic variables on microbiomes has rarely been

107 reported [28,33,37–43].

108   To explore variables that may influence amphibian skin-associated microbial communities at

109 the global scale, we used cutaneous microbiome data from 2,349 post-metamorphic amphibians.

110 We analyzed how multiple factors associated with an amphibian's biology, their abiotic and

111 biotic environment, and their biogeography, related to these communities. Secondly, we

112 investigated bacterial richness and composition of the globally-distributed, American bullfrog

113 (*Lithobates catesbeianus*), to separate intrinsic host-related effects from extrinsic environmental

114 effects shaping the skin microbiome. Lastly, we explored whether our observations agreed with

115 specific, non-mutually-exclusive mechanistic hypotheses that could account for the observed

116 diversity patterns: (1) bacterial relative abundance patterns across important bioclimatic

117 predictors will be associated with bacterial thermal growth optima [44], (2) bacteria with faster

118 growth rates, have a competitive advantage over other bacteria and thus may reduce bacterial

119 richness [45-49], (3) natural environmental fluctuations associated with colder winter temperatures

120 could create opportunities for bacterial turnover and favor dormancy, thus facilitating increased

121 bacterial richness [47], and (4) temperature fluctuations may mediate competitive interactions, such

122 as antibiotic production by microbes, which will influence microbial diversity [48]. To explore

123 these hypotheses, we integrated bacterial community data with information on inferred optimal

124 growth temperatures and quantified predicted functions associated with growth rates, dormancy

125 and antibiotic production. Together these data reveal global patterns of amphibian skin

126 microbiomes and provide mechanistic insights that deepen our understanding of these

127 communities.

128

129

Results

Bioclimate correlates with richness of amphibian skin microbiomes

We built linear mixed models (LMMs) for bacterial richness (number of bacterial sub-operational taxonomic units, sOTUs) and evenness (Simpson's E), from a combination of biotic and abiotic factors including subsets of least-correlated bioclimatic predictors (Supplementary Table 5). Our preferred LMM (Fig. 1A-B; Supplementary Table 7) based on lowest Akaike Information Criterion (AICc) value included five bioclimatic variables, as well as amphibian species richness, latitude and elevation while controlling for four random factors: sequencing center, host habitat class, collection habitat, and host phylogeny (as amphibian family). The biotic variables, host phylogeny and microhabitat, were not included as fixed factors due to their inconsistent and possibly site-driven effects (see below). The highest coefficient value corresponded to minimum temperature of the coldest month (hereafter referred to as Bio6, Supplementary Table 7). A partial effect analysis (Fig. 1B) revealed that bacterial richness negatively related to Bio6, as it did in an independent analysis of this variable (Pearson's $r = -0.301$; $P < 0.001$). In the multivariate context, i.e., controlling for the very strong Bio6 effect, richness is predicted to increase with mean temperature of driest quarter (Bio9), and to decrease with latitude and altitude (Fig. 1B). These variables however have inverse relationships when analyzed independently; richness decreased with Bio9 (Pearson's $r = -0.222$; $P = 4.8^{-26}$) and increased with latitude ($r = 0.175$; $P = 1.0^{-16}$) and altitude ($r = 0.188$; $P = 4.2^{-19}$).

Alternative models included mean annual temperature range (Bio7), which showed a positive correlation with bacterial richness (Supplementary Table 7-8). Given the high correlation between these and many other bioclimatic variables (Supplementary Table 5), these results suggest that richness of amphibian skin microbiomes is higher in more seasonal environments with colder winter temperatures. The preferred LMM for Simpson's E had very low R^2 values for all variables (Supplementary Table 10), confirming that evenness was not strongly influenced by any of the predictors included in our study.

Path analyses provided additional support to our central finding that Bio6 had a strong effect on richness, indicating that (i) elevation strongly influenced Bio6, but only had weak direct effects on bacterial richness, and (ii) Bio6 influenced host richness and host phylogeny, but these two predictors had comparatively weak direct effects on bacterial richness (Fig. 1C-D).

5

162

Bioclimate explains abundance of bacterial taxa in the amphibian microbiome

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Bacterial community similarity based on sOTUs was only marginally influenced by bioclimate

(Table 1), but at phylum level, the relative abundance of Proteobacteria increased, and that of

several other bacterial phyla decreased with Bio6 (Fig. 2A). We used binomial mixed models to

evaluate the effect of bioclimate on the 27 most abundant bacterial genera (greater than 0.5%

relative abundance). Eighteen of these genera were negatively correlated with Bio6 (i.e. increased

in relative abundance with colder temperatures) (Fig. 2B). The standard deviation in slopes

among genera was estimated to be 0.7774, and the main effect of Bio6 overlapped zero

(maximum likelihood estimate:-0.4921, SE=0.3114, Z=-1.580, P=0.114). The effects of Bio6 also

varied with latitude, with an estimated standard deviation of 2.910. We found that the relative

abundance of many bacterial genera across the Bio6 gradient was associated with their thermal

optima (predicted from culture databases of other species in the same genera; see methods), and

therefore probably influenced by bacterial thermo-physiological constraints (see Supplementary

Results).

178

Influences of host phylogeny and microhabitat on microbiome richness

180

Biotic factors, included in LMMs as random factors, contributed to explaining richness of

amphibian skin-associated microbiomes, but revealed only limited globally applicable patterns.

This is apparent from the strong effect of host microhabitat preference (although lower in

coefficient value than bioclimatic factors) when included as a fixed effect (Supplementary Table

13). A detailed analysis of host microhabitat preference suggested this was caused by

idiosyncratic effects in different geographical regions (Fig. 3; see Supplementary Fig. 6 for a

more fine-scale categorization). For example, aquatic frogs had low sOTU richness in the USA

but high values in Panama. As one moderately consistent pattern, arboreal amphibians in five

countries had on average lower bacterial richness than terrestrial amphibians, and these

differences were statistically significant in three countries (Brazil, Madagascar, Panama; P<0.05

in FDR-corrected Wilcoxon U-tests; Fig. 3).

Despite a very strong effect of main host clades (families) in predictor screening (Supplementary

Table 4), host phylogeny based on a non-metric multidimensional scaling (nMDS) proxy, was not

194 a top predictor of bacterial richness when included in LMMs (Supplementary Table 8). At the

195 global scale, however, family-level taxonomy was closely linked to collection site, and therefore

196 its effects could not be reliably disentangled from bioclimatic effects.

197

198 *Bullfrogs mirror native amphibians in microbiome richness and beta-diversity*

199

200 The American bullfrog is globally distributed, allowing for the unique opportunity to explore skin

201 microbiomes across disparate biogeographic regions and to compare bullfrogs to other co-

202 occurring species in dissimilar regions. For this purpose, we collected 139 American bullfrog

203 samples from Brazil, Japan, South Korea, and the USA. Similar to our findings with the full

204 dataset, American bullfrogs had higher bacterial richness in localities with lower minimum

205 temperature of the coldest month (Bio6) (Fig. 1A). It is important to note, however, that bullfrog-

206 specific data do not span the full range of the Bio6 gradient. To examine host effects on patterns

207 of beta diversity we calculated unweighted and weighted Unifrac distances between microbiomes

208 of American bullfrogs and other sympatric amphibians and compared these to distances between

209 allopatric populations of American bullfrogs. Comparisons of both distance metrics showed the

210 same pattern. We found that pairwise distances among bullfrogs and other sympatric amphibians

211 were smaller than pairwise distances among bullfrogs from different sites (Weighted Unifrac,

212 Monte-Carlo approximation; $Z = 11.85$, $P < 2.2^{-16}$ (Fig. 2C)). Additionally, pairwise distances

213 among allopatric bullfrogs were only marginally different from pairwise distances among

214 allopatric non-bullfrog samples. ($Z = 3.07$, p-value < 0.001 (Fig. 2C). Analysis of core

215 communities revealed that no sOTUs were shared among American bullfrogs across continents at

216 ≥70%. Indeed, across the full dataset no sOTUs were shared amongst 90% or 100% of the

217 samples, and only one sOTU was shared among 80% of samples (a *Klebsiella* sp.). For the 27

218 most abundant bacterial genera (Fig. 2B), the effect of Bio6 in binomial mixed models was

219 marginally correlated between the dataset comprising all host taxa and the bullfrog dataset

220 (Pearson's product-moment correlation -0.368, 95% confidence interval of -0.656, and 0.014, P=

221 0.0592; Supplementary Fig. 11). While controlling for species-specific effects, these bullfrog-

222 specific results support that bioclimatic and site-specific factors best explain variation in

223 amphibian skin-associated microbial diversity.

224

225 *Bacterial genes may explain correlation of bacterial richness with bioclimate*

226

227 We used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

228 (PICRUSt) to explore potential hypotheses that could explain observed patterns of bacterial

229 richness across bioclimatic gradients. This tool predicts the functional profile of the entire

230 microbiome through matching 16S amplicon sequences to known bacterial genome data. It is

231 therefore important to note the intrinsic limitations of this predictive framework. On average, 81

232 % (± 18 % SD) of the community was mapped to the Greengenes database required for PICRUSt

233 analyses. Furthermore, amphibian skin microbiomes had suitable Nearest Sequence Taxon Index

234 values (see methods) validating their use in PICRUSt analyses. Using these data, we analyzed

235 average predicted rRNA copy number and relative abundance of two functional categories: (i)

236 dormancy-associated functions, including sporulation, toxin, antitoxin and resuscitation pathways

237 [47,49], and (ii) antibiotic synthesis function, including carbohydrate and lipid metabolism, terpenoid

238 backbone biosynthesis, sterol biosynthesis, aromatic amino acid metabolism, and biosynthesis of

239 secondary metabolites[50]. All the aforementioned gene pathways are well studied[46,52,53].

240 Bacterial taxa are known to code 1–15 ribosomal (rRNA) operons in their genome.

241 Commonly referred to as rRNA copy number, this operon number is a robust and well-studied

242 trait of bacteria that relates to bacterial growth rate and efficiency [48,49]. Previously, rRNA copy

243 number has been identified as an important variable explaining community composition of

244 amphibian skin bacteria during amphibian development [51]. We found that average predicted

245 rRNA copy number was positively correlated with Bio6 (i.e., greater in warmer climates, Kendall

246 rank, r = 0.21, P <0.0001), and that dormancy-associated functional-gene abundance was

247 negatively correlated with Bio6 (i.e. greater in colder climates, Kendall rank correlation, r = -

248 0.27, P < 0.0001; Fig. 4). Lastly, gene abundance of antibiotic synthesis pathways was also

249 negatively correlated with Bio6 (Kendall rank, r = -0.23, P < 0.0001, Supplementary Fig. 8).

250

251 **Discussion**

252

253 This study expands upon previous research by examining macro-ecological patterns of amphibian

254 skin bacteria. Our data revealed that temperature-associated factors, in particular cold winter

255 temperatures and seasonality, consistently correlate with bacterial richness and to a lesser extent

256 with bacterial composition on amphibian skin at the global scale. Our results reflect an inverse

257 latitudinal richness-effect given that a simple regression analysis indicated decreasing richness at

258   lower latitudes. This result likely occurs because temperature-related bioclimatic variables, such

259   as Bio6, and latitude were highly correlated with each other across sampling localities. This

260   pattern is in contrast with what is observed for most free-living macro-eukaryotes, including

261   amphibians [52,53], but mirrors findings of bacterial communities from other environments [6,8–10,54].

262   Only rarely have previous studies on bacterial communities found a conventional latitudinal

263   effect of (higher diversity at lower latitudes [7,55]). For amphibians, we expect that most skin

264   bacteria are environmentally acquired [56,57]. Thus, the observed inverse latitudinal richness-effect

265   could be a function of diversity patterns of environmental substrates[54]. While there are several

266   limitations to our design, such as lack of environmental substrate samples and variability in

267   sampling date, our data provide evidence for a skin-associated diversity gradient in part explained

268   by the latitude-associated temperature regime. This finding is further supported by analyses of

269   American bullfrogs which are globally distributed.

270   Our study also demonstrates that amphibian microhabitat usage influences skin bacterial

271   richness, and that bacterial composition differs among coarse host taxonomic categories, i.e.,

272   amphibian families. While at the local scale, previous studies have demonstrated host-specific

273   patterns in amphibian skin bacteria [28,58–60], the nMDS proxy for amphibian phylogeny herein was

274   less consistently associated with bacterial richness and composition. A low phylogenetic effect on

275   microbiome composition was also found in an in-depth study of amphibian fauna sampled across

276   Madagascar [61]. Amphibian skin physiology and secretions are partly conserved phylogenetically,

277   but perhaps the most influential factors for the skin microbiome are discontinuous across the

278   amphibian tree of life. For instance, multiple unrelated amphibian families have been defined

279   based on important ecomorphological traits such as arboreality (Hylidae, Rhacophoridae,

280   Hyperoliidae) which may influence microbiome characteristics of constituent species.

281   Congruent with other studies [29,30], we found differences in microbial richness among frogs

282   occupying different microhabitats, but these were only partly consistent across this dataset (Fig.

283   3). For various countries and latitudes, our data suggest that skin microbiomes in arboreal hosts

284   are less rich than in terrestrial species, whereas microbiome richness on aquatic hosts varied

285   substantially, as previously seen in Central American amphibians [58]. It is possible that rinsing

286   transient microbes off arboreal species is more efficacious than rinsing microbes off terrestrial

287   species, and/or the higher diversity on terrestrial species reflects higher diversity of the soil

288   environment that they inhabit. Alternatively, amphibian ecology could influence skin-shedding

289   rates, secretion of skin defense compounds and skin structure, which in turn affect bacterial

290   richness. These additional factors may correlate with species identity or major amphibian clades,

9

291   such as families. In light of these independent effects of microhabitat, host species and phylogeny

292   on the amphibian cutaneous microbiome, it is remarkable that the effects of bioclimate are

293   relatively consistent at the global scale.

294   We found only a single prevalent bacterium (*Klebsiella*; [62]) in 80% of our samples, and no

295   core community for American bullfrogs across continents. This finding supports the hypothesis

296   that amphibian skin microbiomes are strongly influenced by local bacterial source communities

297   and abiotic conditions, including temperature. Amphibians can actively thermoregulate to some

298   degree, such as sitting on a warm rock; however, this capacity is unlikely to outweigh the thermal

299   environment on a global scale.

300   Amphibian tissues provide a rich source of resources for microbes. However, as ectotherms,

301   they do not offer protection from seasonal temperature changes. Our results suggest that skin-

302   associated bacteria of ectotherms are under environmental selection and that in cooler climates

303   they are selected upon to withstand temperatures outside of their growth range (*e.g.*, dormancy in

304   cold climates). We hypothesize that natural environmental fluctuations associated with cold

305   winter temperatures could favor dormancy and promote higher diversity by allowing unique

306   bacterial taxa to become active during different times of the year and by allowing bacteria to take

307   advantage of continual microbial turnover. Dormancy, a characteristic of many bacteria, has been

308   comparatively well studied and is highlighted as a factor influencing bacterial biogeography

309   [13,49,63]. In our hypotheses about why we observe an inverse latitudinal diversity gradient, we

310   assert that a combination of bacterial characteristics in these communities may explain this

311   pattern. First, higher predicted rRNA copy numbers, which signifies fast growth, were found in

312   warm, stable thermal environments, suggesting that taxa in these environments are able to out-

313   grow and potentially exclude other bacterial taxa (Fig 4). This result is linked to a reduction in the

314   richness of bacteria on the skin of amphibians in these environments. Second, we hypothesized

315   that a periodic resurgence from the microbial seed bank on amphibian skin, facilitated through

316   dormancy, may bring about higher richness on amphibians inhabiting regions with seasonal

317   thermal changes. In support of this hypothesis, we found that dominant bacterial genera and

318   dormancy genes found on amphibian skin were non-randomly distributed across a temperature

319   (Bio6) gradient (Fig. 4). Indeed, dormant or slow-growing bacteria are more prevalent in

320   environments with seasonal temperature variation, which likely affects nutrient or growth

321   conditions [49]. This hypothesis is consistent with our finding that bacteria with lower thermal

322   growth optima are more abundant on amphibians in regions with colder winter temperatures (see

323   supplemental results). Importantly, estimating thermal optima of bacterial genera within our

324   dataset from databases of bacterial thermal optima is not a direct comparison, and thus does not
325   match the exact conditions of amphibian skin. Further details on these limitations are discussed in
326   the supplement. Alternatively, or as a compounding effect, chemical disturbances (*e.g.*, antibiotic
327   synthesis) in these cooler environments may also play a role in shaping global diversity patterns
328   of bacterial symbionts of ectotherms [64]. We hypothesized that moderate disturbance via chemical
329   antibiotic production from microbiomes may create a more heterogeneous landscape, facilitating
330   open niches, niche specialization and ultimately greater microbial diversity. Again, we found that
331   predicted antibiotic synthesis gene abundances found on amphibian skin were non-randomly
332   distributed across the Bio6 gradient (Supplementary Fig. 8). Direct measurements of functional
333   genes are required to confirm our results obtained from PICRUSt predicted gene functions.
334   Future studies may extend these findings in a variety of ways. As new datasets of both
335   endotherms and ectotherms become available, a meta-analysis including both groups could
336   provide greater insight into either the generality or specificity of our findings. For example, a
337   strong effect of bioclimate on skin microbiomes of endotherms is unlikely given that temperature
338   fluctuations in cold environments are less extreme for microbiomes associated with most
339   endothermic animals, underscoring the importance of studying a broad diversity of animal taxa to
340   understand global host-associated diversity patterns. Future studies could also explore the
341   influence of bioclimate on host-associated microbial communities both within and across
342   vertebrate and invertebrate groups. As microbial genomic databases become better equipped and
343   sequencing of bacterial gene content from whole communities becomes more commonplace,
344   future work could address hypotheses related to ours with sample-specific microbial genomic
345   data. Experimental translocation and temperature manipulations of amphibians could also test for
346   selection of amphibian-associated microbial phenotypes and genotypes.
347   Our results indicate that amphibian skin bacterial composition changes across a bioclimatic
348   gradient, and that bacterial richness per host individual decreases towards warmer, more stable
349   thermal environments. However, due to the compositional nature of sequence data, we
350   acknowledge that changes in abundance of specific bacterial taxa across bioclimatic gradients are
351   influenced by changes of other bacterial taxa. For this reason, our results focus primarily on the
352   global richness patterns across climatic gradients. Future sequencing projects could include a
353   DNA spike-in during sequencing, which enables better estimation of absolute microbial
354   abundances for among-sample comparisons [67].
355   Bioclimatic variables, in particular minimum temperature of the coldest month, and seasonal
356   temperature variation, consistently correlated with cutaneous microbiomes at the global scale.

357   The importance of this aspect of bioclimate in shaping host-associated microbiomes was
358   previously unknown. Our data help explain fundamental questions of microbial biogeographical
359   diversity and offer new insights into how climatic variation may affect host microbiomes. In the
360   face of rapid environmental change around the globe, climatic changes may alter host
361   microbiomes, which in turn, could have consequences on maintenance of host health and
362   selection and evolution of amphibians.
363
364   **Methods**
365
366   *Summary of the metanalysis and newly sampled amphibians*
367
368   We assembled samples from 2,349 individual post-metamorphic amphibians, comprising 27
369   amphibian families (205 species) collected across 12 countries (5 continents) (including 538
370   samples newly sequenced for this study). A summary of amphibian sampling effort across
371   continents is provided in Supplementary Table 1 and Supplementary Fig. 1. All amphibians were
372   swabbed using sterile swabs and DNA directly extracted from these. The V4 region of the 16S
373   rRNA gene was amplified with barcoded primers (515f–806r) and sequenced on Illumina MiSeq
374   platforms (details in Supplementary Materials). Raw sequence data was compiled from newly
375   sequenced datasets and from published studies (Supplementary Table 1). Sequences were quality
376   filtered and further analyzed in Quantitative Insights into Microbial Ecology (QIIME) [65]. sOTUs
377   were determined using the deblur workflow [66](https://github.com/biocore/deblur). After filtering
378   and decontamination procedures, the dataset comprised 45,932,673 reads and an average of
379   19,554 reads per sample. Samples were subsequently rarefied at 2,500 reads per sample and had
380   an average of 277 sOTUs.
381   For analyses, these samples were subdivided into different subsets: (1) the full dataset
382   with samples from all 2,349 amphibian individuals, and (2) a dataset of 1,801 individuals for
383   which host phylogenetic data were available (and excluding American bullfrogs). Additionally,
384   an American bullfrog dataset, comprising 139 samples of this species ranging from -29.5–42.4
385   degrees latitude, was analyzed separately to control for amphibian species identity while
386   exploring biogeographic predictors of skin bacterial communities. We also compiled a
387   standardized data set with 828 individuals, representing a more even sampling of 7–10 samples
388   per host species, for calculating core communities.
389

391

392   To analyze the correlation of abiotic and biotic factors with alpha diversity of amphibian skin

393   microbiomes we used QIIME to calculate two response variables, number of observed sOTUs

394   representing community richness, and Simpson's E representing community evenness. For abiotic

395   predictors, we chose 19 bioclimatic variables as well as absolute latitude, and elevation. In

396   addition, we included biotic variables, such as amphibian species richness and host phylogeny.

397   Furthermore, we controlled for the effect of selected categorical variables including sequencing

398   center, host microhabitat preference and collection habitat of hosts. A full discussion of predictor

399   variables is provided in the supplementary materials (also see Supplementary Table 3).

400   Bioclimatic data was extracted from 1-km spatial resolution climate surfaces for global land areas

401   [67]. Host richness was approximated by extracting amphibian richness data from available maps at

402   10x10 km resolution [68]. Host phylogeny was alternatively represented by (i) a categorical

403   variable using amphibian family as a taxonomic proxy, or (ii) evolutionary divergences, a

404   variable obtained by nMDS. Evolutionary divergences among host species were calculated as

405   patristic phylogenetic distances from an ultrametric timetree recovered from the timetree.org

406   database [69]. To include host phylogeny in models we created a nMDS proxy for host phylogeny,

407   constrained to 1 dimension, on the patristic distance matrix (Kruskal's Stress 1 = 0.023). nMDS

408   values (1-D-ordination explaining 20 percent of the variation) showed no outliers, such that,

409   closely related families have similar values (Supplementary Fig. 12).

410   To assess the effect of all predictors on richness and evenness of bacterial communities (number

411   of sOTUs), we first used Response Screening adjusted for false discovery rate in JMP 13.0 (SAS

412   Institute). Most bioclimatic predictor variables were strongly correlated, and we therefore applied

413   various strategies to compile sets of variables that were strong predictors of the data, least-

414   correlated, or potentially biologically informative based on a-priori assumptions. The selected

415   subsets of variables were implemented in alternative Linear Mixed Models (LMMs) in R using

416   the lme4 package [70,71] and then evaluated on the basis on Akaike Information Criterion (AICc),

417   R^2, and Variance Inflation Factor (VIF) values.

418   Coefficient values for all predictors were obtained through the fixef() function in the nlme

419   package. The sjPlot package was used to created model estimate and effect plots (Fig 1;

420   Supplementary Fig. 2) [72]. All statistical significance is reported using a two-tailed approach. See

421   Supplementary Information for a full description of model selection procedures, predictor

422   variables included in each model, and coefficients and VIF values for each variable.

423   To understand directionality and confirm the relative strength of predictors selected in our

424   LMM model selection procedures, we built six ecologically meaningful path models that

425   included a combination of variables directly or indirectly affecting sOTU richness: Bio6, Bio7,

426   elevation, as well as two biotic variables, host phylogeny and amphibian species richness (Fig

427   1D-E; Supplementary Fig. 4). These confirmatory models were primarily designed to better

428   understand the interactions of the predictor variable found to be most influential (Bio6) with

429   biotic predictors, i.e., host richness and host phylogeny. Path models with the response variable

430   Bio6 averaged by collection site were also performed (Supplementary Fig. 5). From a correlation

431   matrix, estimates of standardized path coefficients with their associated standard errors were

432   derived by Maximum Wishart Likelihood (500 iterations), allowing the identification of

433   significant paths. Latent (unmeasured 'u') variables, corresponding to variance attributed to error

434   and any unmeasured predictors, were estimated for each response variable in the model.

435

436   *Compositional analysis of amphibian skin microbiomes*

437

438   A phylum-level taxonomic summary of amphibian skin microbiomes by host species (n = 205

439   species) within each country is provided in Supplementary Table 2. We used QIIME to calculate

440   beta diversity as weighted Unifrac distances. Factors driving patterns in beta diversity were

441   investigated with Permutational Multivariate Analysis of Variance (PERMANOVA) [73] estimating

442   Pseudo-F and P values with marginal effects.

443   To understand differentiation of American bullfrogs across sites relative to their

444   differentiation from sympatric amphibians we selected samples from only those locations from

445   which bullfrog data were available. We used the make_distance_boxplots.py script in QIIME to

446   calculate pairwise Unifrac distances among different categories of interest, and used Monte-Carlo

447   approximations in R [74], adjusted for false discovery rate, to identify significant differences among

448   these categories.

449   The relative abundance of each of 27 most abundant bacterial genera (overall relative

450   abundance >0.5%) across Bio6 was analyzed by three alternative generalized mixed effect

451   models that differed in their random effect structure (Supplementary Materials). Models were fit

452   using a binomial likelihood with the `glmer` function in the lme4 package and chosen by

14

453   performance based on AICc. We accounted for spatial differences via a random intercept for

454   binned longitude and latitude and allowed the effects of minimum temperature of coldest month

455   (Bio6) to vary among frog genera and latitude via a random slope. Due to the compositional

456   nature of the data, the observation that some taxa decrease along the climatic gradient, while

457   others increase, is just one of many potential underlying dynamics that could yield these

458   taxonomic responses. Indeed, is not possible to distinguish whether there are true changes to the

459   community in both directions, or whether a few taxa are changing substantially in one direction

460   and influencing the proportional abundance of another taxa selection which may appear to change

461   in the opposite direction.

462

463   *Predicted thermal optima, bacterial growth rates, dormancy genes, and antibiotic synthesis*

464   *genes*

465

466   Kendal-Tau rank correlations were run on the relative abundance of all bacterial genera with a

467   representation greater than 0.1% and the strongest predictor variable of bacterial richness, Bio6.

468   For these genera, information on thermal optima of bacteria [44] was obtained from available

469   databases. The thermal optima for bacterial species in the studied amphibian microbiomes are not

470   known directly but were predicted from data of other species in the same genera, studied under

471   laboratory conditions. For a given species, all isolates from a given database were first averaged

472   together to provide one temperature per species. All averaged species temperatures for the genera

473   of interest were then extracted and used for analysis (see Supplementary Methods). This

474   procedure was implemented to minimize over-representation of particular species within a given

475   genus. Mann-Whitney U-tests were then used to compare thermal optima and the variance in the

476   thermal optima between genera that were positively and negatively correlated with Bio6.

477   We used Phylogenetic Investigation of Communities by Reconstruction of Unobserved

478   States (PICRUSt) [50] to estimate bacterial gene function, see Supplementary Methods for details of

479   sOTU clustering and sample selection. In PICRUSt, we normalized the dataset by predicted

480   rRNA copy number, and then predicted the metagenome of each sample to investigate functional

481   abundance of dormancy and antibiotic synthesis pathways. Dormancy analyses included all KOs

482   contributing to sporulation, toxins and antitoxins, and resuscitation [47]. Antibiotic KOs were

483   extracted from KEGG's antibiotic synthesis category. Kendall-Tau correlations were used to

484   explore the relationship between these functions and our main predictor, Bio6. In addition to

485 these functional abundance analyses, we directly explored the average predicted rRNA copy

486 number within a microbiome and how it correlates with Bio6. Predicted rRNA copy number is

487 frequently used to estimate bacterial growth rates [45]. Amphibian samples in this study had

488 sufficient NSTI values for analyses (mean = 0.060 ± 0.034, median = 0.061 ± 0.040). In context

489 and according to a previous study, human-associated samples had the lowest (best) NSTI values

490 (0.03± 0.2), whereas mammalian guts and soil samples had much higher (worse) NSTI values,

491 (0.14± 0.06 and 0.17± 0.02), respectively. Importantly, NSTI values of 0.1 for 16S rRNA marker

492 gene surveys and shotgun metagenomes still resulted in an Spearman r of approximately 0.8 and

493 was considered an accurate gene category assignment[50]. Furthermore, NSTI is an aggregate

494 measure based off of branch length and does not correspond to sequence similarity.

495

496 **Data Availability**

497 A full description of data analyses is provided in Supplementary Materials. Data for all newly

498 sequenced samples is available on the Short Read Archive (Bioproject PRJNA474496). All

499 figures include associated raw data and there are no restrictions on data availability..

500 Correspondence and requests for materials should be addressed to M.V. (), or D. C. W ().

501 **Statement on Competing Interests**: The authors declare no competing interests.

517

518 **Author Contributions**

519 JGK, MCB, VJM, DCW, and MV conceived of the study, coordinated the analyses and wrote the

520 manuscript. JGK, MCB, DCW, GB, MJ, designed and performed data analysis.

521 JGA, AB, MB, LB, AC, CFBH, RNH, WH, MH, JLK, JK, AK, AL, AHL, DM, JJN, RGBP,

522 APT, FCER, EAR, AR, LRS, GVA, BW, JBW, SMW, KZ, IZC contributed materials and data.

523 HA, LA, RG, MJ performed laboratory work, and PJK, RS, and CCT contributed to data analysis.

524 All authors contributed to the development and revision of the manuscript.

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689

692 **Table 1**. PERMANOVA models of beta diversity, showing influence of selected predictors on
693 weighted Unifrac distances among amphibian cutaneous microbiomes. All continuous variables
694 were rescaled before analysis. Models that differ by the host phylogeny proxy included:
695 amphibian family in model A, host phylogeny nMDS variable in model B. PERMANOVA-based
696 P-values of F are <0.001 for both models and all predictors. The R^2 numeric values are colored
697 using a heat map, red signifying higher values compared to the lowest values in blue.
698

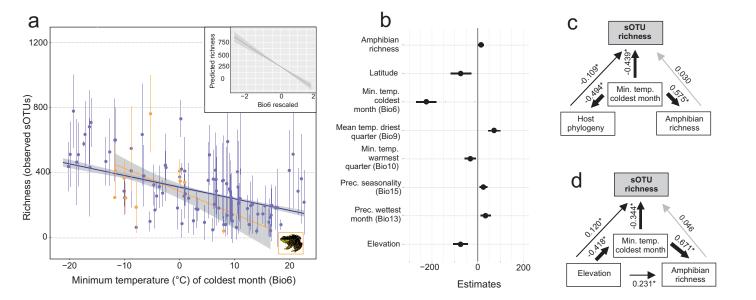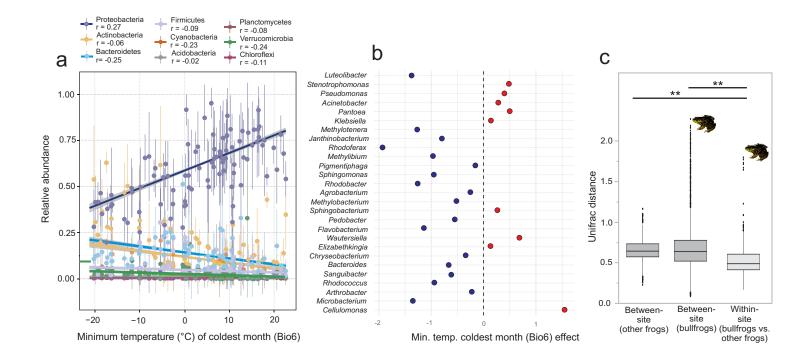| Model A					Model B		
	df	Sum of sqs	*R^2*	*F*	sum of sqs	*R^2*	*F*
Bio4 (temp. seasonality)	1	1.06	0.00278	8.019	0.994	0.0035	7.460
Bio6 (min. temp. coldest month)	1	1.81	0.00475	13.695	1.219	0.00429	9.147
Bio7 (annual temp.range)	1	1.03	0.0027	7.782	1.114	0.00392	8.364
Bio9 (mean temp. driest quarter)	1	0.84	0.0022	6.323	0.461	0.00162	3.462
Bio12 (annual precip.)	1	2.62	0.00688	19.822	2.723	0.00957	20.435
Bio14 (precip. driest month)	1	4.15	0.01089	31.375	3.922	0.01379	29.437
Bio18 (precip. warmest quarter)	1	1.42	0.00372	10.714	1.86	0.00654	13.960
Amphibian richness	1	0.66	0.00175	5.029	0.453	0.00159	3.402
Elevation	1	1.44	0.00379	10.915	1.487	0.00523	11.162
Latitude (absolute value)	1	1.59	0.00417	12.008	1.099	0.00387	8.251
Habitat class	4	7.01	0.01842	13.268	5.775	0.02031	10.835
Amphibian family / host phylogeny nMDS	26/1	17.99	0.04727	5.237	1.4	0.00492	10.504

Captions to Figures

Figure 1. Richness of amphibian skin microbiomes is associated with bioclimate. (a) Plot of raw data for richness vs. non-rescaled values of minimum temperature of coldest month (Bio6), separately for bullfrogs and non-bullfrogs, showing similar trends for these subsets. Inset: partial effects plot from the preferred LMM showing predicted richness values for rescaled Bio6. Gray shading represents 95% confidence intervals, and error bars associated with each point represent standard deviations for each locality. (b) Estimated effect sizes for all included fixed factors in the preferred model. (c) and (d) Path models confirming a strong effect of Bio6 and visualizing how biotic factors, host (amphibian) phylogeny and richness, are equally influenced by bioclimate why having no or only weak effects on bacterial richness. Estimates of standardized path coefficients with their associated standard errors were derived by Maximum Wishart Likelihood (500 iterations). Black arrows indicate statistically significant effects (P<0.05) determined from the Path model analyses, width of arrows is proportional to effect size.
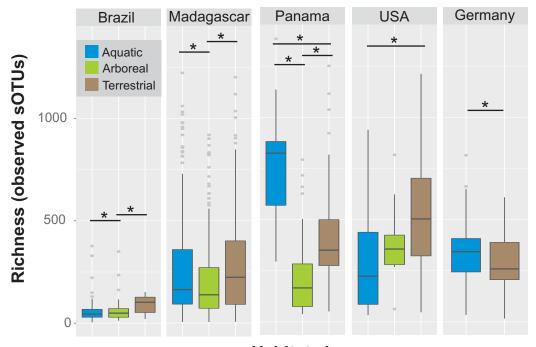
Figure 2. Factors influencing skin microbiome composition. (a) Relative abundances of the top 10 bacterial phyla along the gradient of minimum temperature of coldest month (Bio6). Proteobacteria, Bacteroidetes and Verrucomicrobia exhibited the strongest correlations with bioclimate (r-values are from Kendall Tau correlation tests; all P < 0.001). Gray shading represents 95% confidence intervals. (b) Estimated effects of Bio6 on dominant bacterial genera. Each maximum likelihood estimate is displayed as a point, and the dashed line indicates no effect. Bacterial genera responded differentially, most being more abundant with cold winters (negative values; blue dots) and some being less abundant (positive values; red dots). (c) Boxplot of weighted Unifrac distances among microbiomes of bullfrogs and other sympatric amphibians from four countries (Brazil, Japan, South Korea and USA). Bullfrog microbiomes are significantly more similar to those of sympatric anurans than to those of bullfrogs from other locations. Asterisks indicate significant comparisons (Monte-Carlo approximation, Bonferroni-adjusted, all comparisons **P<0.001). Boxplots display the first quartile, median, third quartile, and maximum values along with outlier data points.

Figure 3. sOTU richness of skin bacterial communities among amphibians occupying different microhabitats, examined for six different countries. Boxplots show mean, standard deviation and range (outliers as gray dots). Significant differences are highlighted by asterisks (pairwise FDR-corrected Wilcoxon U-tests per country, Bonferroni-corrected over the six country comparisons: * P<0.05). The boxplot displays the first quartile, median, third quartile, and maximum values, along with outlier data points. For simplified representation, from the original five-category classification semiaquatic was merged with aquatic, and scansorial with terrestrial, reflecting the main microhabitat of the respective species (full graph in Supplementary Fig. 6).

Figure 4. (a) Average predicted 16S rRNA copies number increases with Bio6 (Kendall rank correlation, r=0.21, P <0.0001), and (b) relative abundance of dormancy-associated gene pathways (summarized for sporulation, toxin/antitoxin, and resuscitation; [49]) decreases with Bio6 (Kendall rank correlation, r=-0.27, P <0.0001). Average copy number and relative gene abundances are calculated per microbial community, and values are represented as medians per sampling site. Gray shading represents 95% confidence intervals.

a

b

c

sOTU richness

-0.109* -0.439* 0.030

-0.494* 0.575*

Host phylogeny | Min. temp. coldest month | Amphibian richness

d

sOTU richness

0.120* -0.344* 0.046

-0.418* 0.671*

Elevation | Min. temp. coldest month | Amphibian richness

0.231*

a

Proteobacteria
r = 0.27

Actinobacteria
r = -0.06

Bacteroidetes
r= -0.25

Firmicutes
r = -0.09

Cyanobacteria
r = -0.23

Acidobacteria
r = -0.02

Planctomycetes
r = -0.08

Verrucomicrobia
r = -0.24

Chloroflexi
r = -0.11

b

Luteolibacter
Stenotrophomonas
Pseudomonas
Acinetobacter
Pantoea
Klebsiella
Methylotenera
Janthinobacterium
Rhodoferax
Methylibium
Pigmentiphaga
Sphingomonas
Rhodobacter
Agrobacterium
Methylobacterium
Sphingobacterium
Pedobacter
Flavobacterium
Wautersiella
Elizabethkingia
Chryseobacterium
Bacteroides
Sanguibacter
Rhodococcus
Arthrobacter
Microbacterium
Cellulomonas

Min. temp. coldest month (Bio6) effect

c

Unifrac distance

Between-site (other frogs)

Between-site (bullfrogs)

Within-site (bullfrogs vs. other frogs)

**

**

a

r = 0.22, p = 0.00017

16S rRNA gene copy number (log) vs Minimum temperature (°C) of coldest month (Bio6)

b

r = −0.27, p = 3.8 × 10^{-06}

Dormancy-associated functional abundance (log) vs Minimum temperature (°C) of coldest month (Bio6)