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Geography, Host Genetics, and Cross-Domain Microbial Networks Structure the Skin Microbiota of Fragmented Brazilian Atlantic Forest Frog Populations

Frog Skin Microbiota Networks

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Abstract

The host-associated microbiome plays a significant role in health. However, the roles of factors such as host genetics and microbial interactions in determining microbiome diversity remain unclear. We examined these factors using amplicon-based sequencing of 175 *Thoropa taophora*

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30 frog skin swabs collected from a naturally fragmented landscape in southeastern Brazil.
31 Specifically, we examined (1) the effects of geography and host genetics on microbiome
32 diversity and structure; (2) the structure of microbial eukaryotic and bacterial co-occurrence
33 networks; and (3) co-occurrence between microeukaryotes with bacterial OTUs known to affect
34 growth of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). While bacterial alpha
35 diversity varied by both site type and host MHC IIB genotype, microeukaryotic alpha diversity
36 varied only by site type. However, bacteria and microeukaryote composition showed variation
37 according to both site type and host MHC IIB genotype. Our network analysis showed the
38 highest connectivity when both eukaryotes and bacteria were included, implying that ecological
39 interactions may occur among Domains. Lastly, anti-*Bd* bacteria were not broadly negatively co-
40 associated with the fungal microbiome, and were positively associated with potential amphibian
41 parasites. Our findings emphasize the importance of considering both Domains in microbiome
42 research, and suggest that for effective probiotic strategies for amphibian disease management,
43 considering potential interactions among all members of the microbiome is crucial.
44
45 **Keywords:** amphibian, skin microbiome, microbial networks, Brazil's Atlantic Forest
46
47 **Background**
48 The host-associated microbiome has recently captured the attention of wildlife disease
49 researchers seeking to understand and predict disease-associated wildlife declines. In particular,
50 skin microbiome research is burgeoning in the field of amphibian disease. A majority of
51 amphibian disease studies focus on chytridiomycosis caused by the pathogenic fungus
52 *Batrachochytrium dendrobatidis* (*Bd*). *Bd* has been linked to severe amphibian declines around
53 the world since at least the 1970s [1–4]. In some regions, however, no declines have been
54 observed despite the presence of *Bd*. For example, plethodontid salamanders in the Eastern
55 United States showed no evidence of disease-associated declines despite the presence of *Bd* in
56 the environment [5]. In a series of foundational studies, many of which were performed *in vitro*,
57 the presence of certain bacteria cultured from salamander skin was correlated with reduced
58 disease risk [6–8]. Further studies showed that this was also the case in some anurans [9–11] and
59 pointed to antifungal bacterial metabolite production as the main mechanism behind this
60 correlation [7,12,13]. These findings among others gave rise to interest in characterizing

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61 amphibian skin microbiome bacteria as a correlate of *Bd* susceptibility, and in using "probiotic
62 strategies" (manipulating amphibian skin bacteria) to mitigate disease-associated amphibian
63 declines in the wild [13–16].

64 However, the potential non-target impacts of manipulating bacteria are difficult to
65 predict, as much remains to be understood about the diversity and assembly of the overall
66 amphibian skin microbiome aside from the intensively-studied *Bd*-inhibitory bacteria. In
67 particular, little is known about the ecological roles of non-bacterial taxa (but see [17,18]), or
68 interactions between bacteria and skin microbial eukaryotes other than *Bd*. A diversity of
69 microeukaryotes including fungi, microscopic metazoans, and protists have been identified on
70 amphibian skin using high-throughput sequencing [19,20]. In previous studies, fungi comprised
71 the dominant eukaryotic taxon on adult amphibians [17], and exhibited greater efficacy in Bd
72 inhibition compared with bacteria [18]. Although little is known about the ecological roles of
73 these fungi in the amphibian skin microbiome, symbiotic fungi are known to aid in protection
74 against fungal pathogens in other host-microbial systems [21,22]. Fungi in the amphibian skin
75 microbiome may also serve as hyperparasites, *i.e.*, parasites of pathogens/parasites. For example,
76 the cryptomycete fungus *Rozella* parasitizes chytrid fungi [23]. Less is known about the
77 symbiotic roles of host-associated protists, although microbiome eukaryotes on the whole have
78 been shown to impact health [24,25] and immune function [26] in mammals. Thus, microbiome
79 eukaryotes may significantly impact disease susceptibility in vertebrates, including amphibians.
80 Without an understanding of the interactions between microbiome eukaryotes and bacteria, it is
81 impossible to predict the potential microbiome-wide effects of proposed *Bd* control measures
82 that involve manipulating the members of these communities.

83 In addition, few studies to date have examined the genetic mechanisms that determine
84 host-associated microbiome assembly and diversity. From research on mammals, it is known that
85 microbiome assembly and diversity can co-vary with overall host genetic diversity [27] as well
86 as host immunogenetics [28,29], with the latter relationship hypothetically resulting from
87 interactions between immune cells and microbes including commensals and pathogens. In
88 amphibians, previous studies have demonstrated that geography, host identity, and
89 developmental stage can influence skin microbiome diversity [30–32]. Yet only a single study to
90 date has linked amphibian skin microbiome diversity with overall host genetic variability [32].
91 Although no studies have directly examined the relationship between immunogenetics and

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92 microeukaryote diversity or structure in amphibians, an experimental study on the laboratory
93 model frog *Xenopus laevis* suggested that MHC (major histocompatibility complex)
94 immunogenes may determine the ability of hosts to tolerate different microbes [33]. The
95 relationship between immunogenes and the amphibian host-associated microbiome remains to be
96 explored, and is increasingly relevant for wild amphibian populations threatened by emerging
97 disease.

98 In a number of amphibian species, genetic diversity has been compromised due to
99 anthropogenic habitat fragmentation [34]. Although it is unknown to what extent habitat
100 fragmentation impacts the amphibian skin microbiome, genetic erosion in fragmented amphibian
101 populations has been observed at neutral loci as well as immunogenetic regions [20] which may
102 have implications for microbiome structure [28]. In addition, fragmentation may cause a decline
103 in microbial transmission, which in turn may alter microbial interactions and networks in host-
104 associated microbiomes [35,36]. However, the effects of habitat fragmentation on wildlife are
105 subject to time lags [37]; genetic erosion resulting from inbreeding may not be detectable for
106 several generations following habitat fragmentation, making the impacts on genetics and related
107 factors difficult to detect in recently fragmented populations. Historically fragmented
108 populations offer an opportunity to examine the effects of genetic erosion on the microbiome and
109 broader animal health.

110 We evaluated the effects of long-term habitat fragmentation on the amphibian skin
111 microbiome using a historically fragmented model system in the Brazilian Atlantic Forest. This
112 system consists of dozens of land-bridge islands, which were naturally separated from the
113 mainland 12,000-20,000 years ago via sea level rise [38] and thus represent ancient forest
114 fragments. Contemporary insular frog populations were once part of contiguous coastal
115 populations, and are now functionally isolated to the islands [39,40]. Using this geographic
116 setting, we examined the impacts of geography and host genetics on skin microbiome diversity
117 and community structure in a single frog species, *Thoropa taophora* ([Cycloramphidae]), found
118 across coastal mainland and island sites. The island populations of *T. taophora* have experienced
119 fragmentation-induced genetic erosion at both neutral and immunogenetic loci [20,40]. Previous
120 work also showed that island and coastal mainland populations exhibited low *Bd* prevalence and
121 very low zoospore loads, suggesting low *Bd* susceptibility in this species [20]. Commonly it is
122 hypothesized that low *Bd* susceptibility is associated with the presence of anti-*Bd* microbes.

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123 Therefore this system offered the opportunity to ask a number of important questions about the
124 relationships between geography, host genetics, bacteria and eukaryotes in the skin microbiome,
125 within the context of potential protection from *Bd*.

126 We used amplicon-based high-throughput DNA sequencing to analyze bacterial and
127 eukaryotic microbes found in skin swab samples collected from *T. taophora* frogs across coastal
128 mainland and island sites. We examined the relationships between microbes and geography and
129 genetics, as well as the connections of microbes across domains (bacteria vs. eukaryotes). We
130 compared bacteria we recovered from *T. taophora* skin swabs to a database of amphibian
131 microbiome bacterial isolates that have been previously tested for anti-*Bd* activity in challenge
132 assays. Because the mechanism by which these bacteria inhibit *Bd* is not specific to the
133 interaction, but works through metabolites produced by bacteria that have broad anti-fungal
134 activity [10], this database may be used as a proxy for antifungal inhibitory compound
135 production. We used this database to identify which of the bacteria on *T. taophora* skin matched
136 bacterial OTUs that were previously identified as *Bd* inhibitory, *Bd* enhancing, and having no
137 effect on *Bd*, and evaluated whether these categories explained co-occurrence patterns between
138 these bacteria and (non-*Bd*) microeukaryotes found in the *T. taophora* microbiome. Our study
139 was designed to address the following research questions: (1) Does geography and/or host
140 genetic diversity structure the microbiome community? (2) How is bacterial diversity and
141 community assembly related to microeukaryotic diversity and community assembly in the skin
142 microbiome? (3) Do bacteria that affect *Bd* growth have predictable associations with other skin
143 microeukaryotes that could result in unintended consequences if probiotic anti-*Bd* bacteria are
144 applied to frog skin?

145

146 **Methods**

147 *Study system and field sampling*

148 The focal species for this study is *Thoropa taophora,* a cycloramphid frog with a unique
149 tolerance for coastal habitat that allows a wide distribution across the coastal Atlantic Forest of
150 São Paulo State [41]. Adult *T. taophora* frogs (n=175 total) were sampled from each of ten study
151 populations: seven island populations and three coastal mainland populations (Fig. 1, Table 1;
152 SISBio collection permit 27745-13). Genetic diversity is lower in island *T. taophora* populations
153 relative to coastal mainland populations, both at neutral (microsatellite) loci [40] as well as at the

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154 MHC IIB immunogenetic locus [20]. To examine how host genetics impact skin microbiome
155 diversity, skin swab samples were analyzed from the same individuals that were previously
156 genotyped at MHC IIB (see [20]). Prior to tissue collection, frogs were thoroughly washed with
157 sterile (autoclaved) distilled water and then swabbed on the ventral surface using standard
158 protocols that minimize cross-contamination [42]. Swabs and tissue samples were stored in 70%
159 ethanol in sterile microcentrifuge tubes before laboratory processing. DNA was extracted from
160 swabs using a Qiagen DNeasy Blood and Tissue kit, and DNA extracts were stored at -20°C
161 prior to further molecular work.
162
163 *Microbiome Sequencing and Bioinformatic Processing*
164 Individual swab DNA extracts were PCR-amplified, pooled and sequenced on the
165 Illumina MiSeq platform (250 bp paired-end reads) in two assays: (1) barcoded 16S primers
166 515F and 806R [43] were used to examine bacterial diversity; and (2) barcoded 18S v4 primers
167 TAReuk454FWD1 and TAReukREV3 [44] were used to examine microeukaryote diversity. 16S
168 libraries were constructed at the Universidade Estadual Paulista (BR) and sequenced at the
169 Tufts University Core facility (USA) while 18S library preparation and sequencing were
170 performed at the University of Michigan (USA). Negative (template-free) controls were run
171 simultaneously with each sequencing library to ensure there was no contamination from PCR or
172 sequencing reagents.
173 Sequences were quality-filtered and processed using the Quantitative Insights into
174 Microbial Ecology (QIIME) MiSeq pipeline using default settings [45]. As no mock community
175 was included as a positive sequencing control, low abundance OTUs were filtered from the
176 dataset using a conservative abundance threshold (<0.005% of all reads) [46]. Sequences were
177 clustered into operational taxonomic units (OTUs) using a 97% similarity threshold and
178 compared against reference databases to assign taxonomy (GreenGenes 13.8 and RDP search for
179 16S, Silva 119 and BLAST search for 18S). Chimeras were identified and filtered using
180 UCHIME2 [47]. 16S sequences from chloroplasts and mitochondria and 18S sequences assigned
181 to frog or other non-target non-microbial species (*e.g.*, Streptophyta) were filtered from the
182 dataset. 16S sequences were rarefied to 2000 per sample and 18S sequences were rarefied to
183 1000 per sample based on visual examination of read accumulation curves and plots of
184 rarefaction values versus number of samples retained across sites (Fig. S1). These sequence

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185 threshold values for rarefaction were selected to balance achieving an adequate representation of
186 microbial communities with retaining sufficient site sample sizes, and are within the range of
187 similar previous studies that have used 1000-2000 as threshold values for 16S v4 datasets
188 [48,49].

189 To determine whether potential ecological relationships between bacteria and
190 microeukaryotes reflect potential ecological relationships between bacteria and *Bd*, bacterial
191 OTU representative sequences from the *T. taophora* samples were compared against a reference
192 sequence database of bacteria previously isolated from amphibian skin and categorized
193 according to effects on *Bd* growth in co-culture experiments [50]. The BLAST algorithm was
194 implemented and an E-value threshold of $E < 1e\text{-}20$ was used to identify OTU matches with the
195 reference database. Matching *T. taophora* skin bacteria were categorized as *Bd* enhancing, *Bd*
196 inhibiting, or having no effect on *Bd* growth.
197

198 *Data Analysis*

199 To evaluate overall patterns of microbiome alpha diversity in the rarefied 16S and 18S
200 datasets, Spearman's correlation tests were implemented in R (vrs. 1.7-11; [51]) and performed
201 between 16S and 18S alpha diversity according to (1) OTU richness and (2) phylogenetic
202 diversity calculated in QIIME. To evaluate the relationships between alpha diversity and
203 geography and host genetics, two-way ANOVA tests were performed in R (vrs. 1.7-11; [51]).
204 Separate two-way ANOVA tests were run for each response variable with four ANOVAs run in
205 total. The four response variables were calculated in QIIME and consisted of: (1) OTU richness
206 for bacteria (16S), (2) phylogenetic diversity for bacteria, (3) OTU richness for microeukaryotes
207 (18S), and (4) phylogenetic diversity for microeukaryotes. Each response variable was tested
208 against the factors of site type (island vs. coastal mainland) and MHC IIB genotype (homozygote
209 vs. heterozygote). Two-way ANOVA models were initially run for each response variable with
210 an interaction between site type and MHC IIB genotype, and if the interaction term was non-
211 significant, the model was re-run with an additive effect between factors instead. Assumptions of
212 linear models were confirmed with visual examination of residuals vs. fitted values plots and
213 normal Q-Q plots. Response variables were natural log-transformed to meet assumptions of
214 equal variance and normality. Abundance-based diversity indices (*e.g.*, Shannon's Indices) were
215 not analyzed because using sequence reads as a proxy for abundance can be problematic due to

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216 primer amplification bias and variable copy number of 16S and 18S across microbial taxa [52–
217 55].

218 To evaluate microbial community structure across geography and host population
219 genetics, beta diversity was calculated using the unweighted (*i.e.*, does not account for
220 reads/sequence abundance) UniFrac (phylogeny-based) method in QIIME. Bacterial and
221 microeukaryotic beta diversity were analyzed in separate Mantel tests against geographic
222 distance, neutral genetic distance (F_{ST} calculated from microsatellites), and immunogenetic
223 distance (F_{ST} calculated from MHC IIB sequences) among populations. Mantel tests were
224 implemented in the ade4 package of R [51,56–58].

225 To examine associations between microbial taxa and geography or host frog MHC IIB
226 genotype, data were statistically analyzed and visualized using packages implemented in Python
227 (vrs. 2.7.13) using Matplotlib [59,60]. Associations between microbial communities and
228 geography or frog MHC IIB genotype were determined by simulating an expected null
229 (randomized) distribution of host frog microbiomes. To create the null distribution, a two-
230 column data table was first created with column 1 being the site type (island or coastal) or MHC
231 IIB genotype (heterozygous or homozygous) of a host frog and column 2 being a single
232 microbial OTU found on that frog. After the data table was populated for all frogs and microbial
233 OTUs in the dataset, column 2 (microbial OTU) was held constant while column 1 (site type or
234 frog genotype) was shuffled randomly. This was repeated 1000 times to create two sets of
235 random microbial occurrence distributions, one for analysis of microbial associations with site
236 type and a second for analysis of microbial associations with host frog genotype.

237 Co-occurrence between microbial OTUs within and among domains (Bacteria vs.
238 Eukaryotes) was analyzed with a third null distribution of microbial communities. Because of
239 potential effects of site on microbial presence and community structure (*e.g.*, some microbes
240 only co-occur on frogs because the microbes themselves solely occur at the same subset of
241 sampling sites) and site-MHC IIB genotype interactions (as homozygotes and heterozygotes are
242 not evenly distributed across sites or site types; Table 1), an expected null distribution of
243 microbes accounting for site-specific presence/absence of each microbe was created. This null
244 distribution of microbes was achieved through within-site randomization using MCMC edge
245 swapping, a standard method for network datasets [61–63]. This method allows any
246 configuration to be reached from any starting point, and allows for even sampling along all

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247 allowed states as forward and backward swaps are equally likely. To achieve this, first, two
248 microbe-frog pairs were randomly selected (each pair consisting of a single randomly selected
249 microbial OTU found on a single randomly selected frog). Microbial OTUs were then swapped
250 between the selected frogs when three criteria were met: (1) the frogs were different individuals
251 with the same MHC IIB genotype (either both homozygous or both heterozygous); (2) the OTUs
252 were different from one another; and (3) neither frog already hosted the microbe it would receive
253 via the swap. Microbe swapping was performed with 1000 repetitions for each frog-microbe pair
254 to construct a single set of randomized frog-microbe pairs. The distribution of co-occurrences
255 under this null model was estimated using 320,000 such randomized sets.
256 To test whether hypothesized bacterial effects on *Bd* extend to diverse microeukaryotic
257 members of the microbiome, bacterial OTUs that matched the Woodhams et al. (2015) database
258 were binned according to their hypothesized ecological significance with regard to *Bd* (*Bd*
259 inhibitory, *Bd* enhancing, or no effect on *Bd*). The co-occurrences of bacteria within each
260 category with microbiome eukaryotes were then compared with the third null distribution of
261 microbial OTUs.
262 For all microbial association/co-occurrence analyses, the probability of non-random
263 microbial association/co-occurrence (*p*) was calculated by comparing observed versus expected
264 (null/randomized) counts of microbial association/co-occurrence. *P*-values were evaluated at
265 significance levels of $\alpha = 0.05$ and 0.01with correction applied to account for multiple
266 comparisons [64]. Using the results of the tests of co-occurrences within and among all microbial
267 taxa, microbial networks for bacteria only, microeukaryotes only, and for all bacteria and
268 microeukaryotes were visualized using Matplotlib [59,60].
269
270 **Results**
271 *Overall patterns of microbiome diversity*
272 There were 303 bacterial OTUs and 845 microeukaryotic OTUs recovered across all
273 samples after filtering and rarefaction. Bacterial phylogenetic diversity was positively correlated
274 with microeukaryotic phylogenetic diversity across all samples (Spearman's rank correlation, $\rho =$
275 0.28, $p < 0.01$; Fig. 2A). There was no significant correlation between bacterial and
276 microeukaryotic OTU richness (Spearman's rank correlation, $p > 0.05$).

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277 Proteobacteria, and particularly Gammaproteobacteria, were the most dominant bacterial
278 taxon across all samples, both by number of OTUs and sequence reads (Fig. 3A&B).
279 Gammaproteobacteria also formed a core bacterial microbiome (*i.e.*, this taxon was abundant
280 across samples; Fig. 3C). Among the eukaryotic microbiota, fungi were dominant by both
281 number of OTUs and sequence reads (Fig. 3D&E). No core group of eukaryotic taxa was
282 recovered, though some fungal OTUs were common and found in approximately 50% of samples
283 (Fig. 3F). These common fungal OTUs were members of the Ascomycota, Basidiomycota, and
284 unclassified fungi.
285
286 *Associations between geography, host genetics, and the skin microbiome*
287 Patterns of alpha diversity across site types and MHC IIB genotypes varied among
288 bacteria and microeukaryotes. In all analyses, there were no statistically significant interactions
289 between site type and MHC IIB genotype, and all response variables (16S OTU richness, 16S
290 PD, 18S OTU richness, 18S PD) were natural log-transformed to meet the assumptions of
291 ANOVA tests. Bacterial phylogenetic diversity (PD) varied by both MHC IIB genotype and by
292 site type (Two-way ANOVA, $F(2, 117) = 4.536$, $p = 0.01$, site type $p < 0.05$, MHC IIB genotype
293 $p = 0.01$; Fig. 2B). Average bacterial PD among island frogs was 6.11 compared with average
294 PD of 5.25 for coastal mainland frogs, while average bacterial PD among MHC IIB
295 heterozygotes was 6.64 compared with an average of 5.58 in homozygotes. Microeukaryotic PD
296 did not vary by either site type or MHC IIB genotype (Two-way ANOVA, $F(2, 139) = 2.587$, $p >$
297 0.05). Bacterial OTU richness varied only by MHC IIB genotype (Two-way ANOVA, $F(2, 117)$
298 $= 3.289$, $p < 0.05$, site type $p > 0.05$, MHC IIB genotype $p = 0.01$; Fig. 2C) with MHC IIB
299 heterozygotes hosting 43.0 bacterial OTUs on average compared with 34.3 average bacterial
300 OTUs on MHC IIB homozygotes. In contrast, microeukaryotic alpha diversity varied by only
301 site type for OTU richness (Two-way ANOVA, $F(2, 139) = 5.062$, $p < 0.01$, site type $p < 0.01$,
302 MHC IIB genotype $p > 0.05$). On average, mainland coastal frogs hosted 48.1 microeukaryotic
303 OTUs compared with 32.6 average microeukaryotic OTUs on island frogs.
304 Community composition showed variable patterns. Beta diversity did not vary by
305 geographic distance or either measure of genetic distance (microsatellite F_{ST} and MHC IIB F_{ST})
306 according to Mantel tests (all $p > 0.05$). However, when OTUs were compared with random
307 expectations, significant associations with site type and MHC IIB genotype were observed for

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308 both bacteria and microeukaryotes (Fig. 4). Among the bacteria, Cyanobacteria and
309 Alphaproteobacteria showed significant positive associations with coastal mainland sites
310 regardless of host MHC IIB genotype, while four bacterial groups (Bacteroidetes, Firmicutes,
311 Fusobacteria, unclassified Proteobacteria, and Spirochaetes) showed positive associations with
312 island sites regardless of genotype (Fig. 4A). Among the microeukaryotes, Fungi were
313 significantly positively associated with island sites regardless of host genotype (Fig. 4B). Coastal
314 mainland MHC IIB heterozygotes showed significant positive associations with Ciliates,
315 Helminths, unclassified microeukaryotes, Rhizaria, and Stramenopiles, while mainland
316 homozygotes were significantly positively associated with Ichthyosporea and Nucleariids. Island
317 MHC IIB homozygotes showed significant positive associations with Algae and Apicomplexans.
318
319 *Microbial networks within and among domains*
320 Separate networks were constructed for bacteria and microeukaryotes based on tests of
321 co-occurrence between OTUs within and among taxonomic groups across Domains (Fig. S2). A
322 dominant bacterial network assembled that consisted of 9/16 bacterial taxa: Bacteroidetes,
323 Firmicutes, and Deltaproteobacteria were at the center of the formed network, with connections
324 to Deferribacteres, Fusobacteria, Spirochaetes, Verrucomicrobia, unclassified Proteobacteria, and
325 unclassified Bacteria (Fig. S3). The remaining groups did not form any connections, although
326 there were strong connections formed among OTUs within the Gammaproteobacteria. Within the
327 microeukaryotes, no network connections formed among the 21 taxonomic groups, but there
328 were significant connections between OTUs within the Algae and Rhizaria (Fig. S4).
329 The cross-Domain network analysis resulted in a number of previously unconnected taxa
330 becoming connected with others, with 4/6 previously unconnected bacterial groups and 8/21
331 previously unconnected eukaryotic groups becoming connected to taxa across Domains (Fig. 5).
332 Three networks formed, the largest of which consisted of the previously constructed bacteria-
333 only network with additional connections between bacterial taxa and six eukaryotic taxa: two
334 fungal groups (Zoopagomycota and unclassified fungi) and three protist groups (Helminths,
335 Nucleariids, and Stramenopiles). The second largest network formed between three bacterial
336 groups and two eukaryotic groups, all five of which had previously been unconnected in the
337 bacteria-only and eukaryote-only networks: Actinobacteria, Cyanobacteria, Alphaproteobacteria,

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338 Basidiomycota, and Algae. The third and smallest network consisted of Gammaproteobacteria
339 and Rhizaria.
340
341 *Associations between microbiome eukaryotes and bacteria reported to inhibit, enhance, or have*
342 *no effect on* Bd *growth*
343 When compared to bacterial OTUs that had been previously tested against Bd in co-
344 culture inhibition experiments [50], nearly half (45%) of *T. taophora* skin bacterial OTUs
345 showed a match at the BLAST E-value threshold of E < 1e-20 (Fig. S5). Tests of co-occurrence
346 between eukaryote groups and these matched bacterial OTUs revealed that enhancing, inhibitory,
347 and no effect do not generally reflect the associations of these bacteria with fungi specifically or
348 microeukaryotes generally (Fig. 6). *Bd* enhancing bacteria were significantly negatively
349 associated with the Ascomycota and Basidiomycota fungi as well as Stramenopiles. *Bd*
350 inhibitory bacteria showed significant positive associations with the Choanoflagellates, and
351 significant negative associations with the Basidiomycota fungi and other unclassified fungi.
352 These also showed non-significant positive associations with Cryptomycota fungi,
353 Ichthyosporeans, and Nucleariids, and negative associations with Apusozoa. Finally, bacteria
354 that were previously found to have no effect on *Bd* were significantly positively associated with
355 Ascomycota fungi, Choanoflagellates, Ciliates, and Rhizaria.
356
357 **Discussion**
358 *Amphibian skin microbiomes exhibited high microeukaryote diversity and were dominated by*
359 *Proteobacteria*
360 In this study, we examined amphibian skin microbiome structure and diversity with
361 respect to geography and host genetics. In analyzing both bacterial and microeukaryote OTUs,
362 we recovered microbial associations with geographic and host genetic factors, as well as
363 unexpected patterns of microbial co-occurrence across Domains. The diversity of
364 microeukaryotes we recovered is higher than previous reports from wild frogs: we recovered 845
365 OTUs in our study compared with *e.g.*, 255 OTUs on *Rana cascadae* [19] and 500 OTUs on
366 *Anaxyrus boreus* [14]. In contrast, the level of bacterial diversity we recovered is lower than
367 previous reports: we recovered 303 bacterial OTUs compared with ~600 OTUs on *Rana italica*
368 [54], although we note that this could be due to different filtering thresholds. Our recovery of

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369 bacteria from 11 phyla is within the range of taxonomic diversity previously recovered from
370 amphibian skin, with for example 10-18 bacterial phyla reported from three species [65]. Our
371 analysis showed that total microeukaryotic and bacterial phylogenetic diversity were positively
372 correlated across all samples, which is a novel finding to our knowledge.

373 Proteobacteria, and in particular Gammaproteobacteria, was the most dominant bacterial
374 phylum on *T. taophora* skin across all study populations, in terms of both OTUs and relative
375 abundance (Fig. 3). This is similar to findings from bacterial microbiome studies of other tropical
376 post-metamorphic anurans [66–70]. Proteobacteria are known to be common in a variety of
377 environments, and contain bacteria that can be pathogenic in amphibians [71]. The dominance of
378 Proteobacteria on amphibian skin has been hypothesized to result from a protective symbiosis
379 between bacteria and amphibians, as many members of the Proteobacteria produce anti-*Bd*
380 metabolites [72,73]. The presence of a high number of Proteobacteria on *T. taophora* skin could
381 potentially contribute to its low apparent susceptibility to *Bd* [20]. It is important to note however
382 that the present study is correlative; without experimental manipulations it is difficult to pinpoint
383 which factors (*e.g.*, the physiology of the skin, mucosal biochemistry, host-microbial
384 evolutionary processes, or interactions with the saline coastal environment) are responsible for
385 the overwhelming dominance of Proteobacteria on *T. taophora* skin.

386 Although bacteria were less diverse than microeukaryotes in our samples, bacteria could
387 nevertheless dominate the skin microbiome according to microbial biomass, which we did not
388 quantify in our study. Sequence reads are sometimes used to estimate relative abundance, but this
389 has been shown to be an unreliable measure due to known sequencing biases among microbial
390 taxa [74]. It is possible that taxa representing fewer OTUs (*i.e.*, bacterial species/strains)
391 represent a higher proportion of microbial biomass, and this should be considered in
392 interpretations of our results. We recommend that future research to address the relationship
393 between microbial diversity and abundance employ high-throughput sequencing alongside
394 quantitative analyses, for example quantitative PCR.
395

396 *Microbiome diversity and structure varied with site type and host immunogenetics*

397 Immunogenotype at the MHC IIB locus was associated with alpha diversity of bacteria
398 on *T. taophora* skin such that MHC IIB heterozygotes hosted a greater number of bacterial OTUs
399 and higher bacterial phylogenetic diversity. Site type (*i.e.*, island vs. coastal mainland) was also a

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400 significant factor in alpha diversity for both bacteria and microeukaryotes (although only for
401 microeukaryote OTU richness, not for phylogenetic diversity). However, beta diversity was not
402 associated with geographic distance or genetic structure of populations at either neutral genetic
403 markers or the MHC IIB immunogenetic locus. These results differ from previous studies on
404 amphibians, in which there were similarly no geographic effects on amphibian skin microbiome
405 structure, but there was a significant association with metapopulation genetic structure [32,75].
406 One possible explanation for the discrepancy between our results and the results from previous
407 studies (barring host identity factors) is that our study populations represent a set of connected
408 mainland populations contrasted with a set of island populations that have been isolated for
409 12,000-20,000 years. The lack of association with genetic differentiation in our populations may
410 be due to this relatively long period of divergence relative to other studies, isolation between
411 island sites resulting in different environmental availability of microbes, or simply environmental
412 differences between island and mainland sites.

413 As microbial diversity was lower in island frogs, this suggests that microbiome diversity
414 may be influenced by genetic diversity: island populations are genetically impoverished, and
415 possess lower microeukaryotic and bacterial diversity relative to coastal mainland populations.
416 Unlike in bacteria, microeukaryotic alpha diversity was not statistically associated with MHC
417 IIB genotype. However, several microeukaryote groups showed significant associations with
418 MHC IIB genotype. Interestingly, MHC IIB homozygotes showed significant positive
419 associations with potential amphibian parasites including Ichthyosporeans in coastal mainland
420 populations, and fungi and Apicomplexans in island populations. These patterns may indicate
421 associations between genetic factors and assembly of the microbiome such that MHC IIB
422 molecules mediate the prevalence of beneficial microbes in addition to pathogenic ones.

423 Taken together, our results imply that host genetics, and specifically MHC IIB genotype,
424 may play a significant role in determining overall microbiome diversity and structure. Although
425 MHC genotype is thought to primarily associate with immune defense against pathogens, results
426 from laboratory and field studies suggest that MHC genotype and allelic composition can impact
427 amphibian host-associated microbial assemblages more broadly [33,76]. The positive
428 associations we found between MHC IIB heterozygosity and bacterial microbiome diversity, as
429 well as with overall microbiome community composition, suggest there may be unknown
430 relationships between MHC molecules and host-associated microbes beyond antagonistic

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431 interactions between immune molecules and pathogens. However, further research is needed to
432 confirm these associations in other species, and determine the contributing mechanisms.
433
434 *Cross-Domain co-occurrence in the amphibian skin microbiome network*
435 Our microbiome network analyses revealed a number of notable patterns. When analyzed
436 separately, the bacterial network consisted of one major group, while no microeukaryote groups
437 formed significant connections with one another. However, in the overall microbial network, a
438 number of microbial groups exhibited cross-Domain co-occurrence: a majority of previously
439 unconnected bacterial groups (4/6) and a number of previously unconnected microeukaryote
440 groups (8/21) became connected in the overall microbiome networks. To our knowledge, ours is
441 the first study to demonstrate these positive cross-Domain network connections in the amphibian
442 skin microbiome.
443 One important implication of this result is that previously undocumented ecological
444 interactions may exist between microbiome bacteria and eukaryotes that in turn may significantly
445 impact microbial assembly. It is currently unclear how widespread cross-Domain associations
446 are, as previous studies that have examined both bacteria and microeukaryotes on amphibian skin
447 have focused on taxon-specific associations, namely between *Bd*-inhibitory bacteria and fungi
448 [17], or between *Bd* and either bacteria or microeukaryotes [19]. While potential antagonistic
449 interactions with *Bd* have been the focus in cross-Domain research on the amphibian skin
450 microbiome, microbial interactions can occur across the spectrum of biological symbioses
451 (reviewed in [64]). Mutualistic interactions between bacteria and microeukaryotes have been
452 documented in other systems; for example, mycorrhizae-helper bacteria are known to indirectly
453 facilitate plant-fungal interactions in the multitrophic mycorrhizal complex [78]. An alternative
454 explanation for our network analysis results is that bacteria and eukaryotes positively co-occur
455 due to co-filtering via specific host, environmental, or other exogenous factors unrelated to
456 microbial interactions. Further research is needed on cross-Domain microbial co-occurrence
457 patterns, microbial interactions, and implications for amphibian host health.
458
459 Bd *inhibitory and enhancing bacteria have variable effects on microbiome fungi and protists*
460 Our dataset included a number of bacteria previously shown to inhibit *Bd*, which have
461 been generally termed "antifungal" [43]. However, bacteria with previously demonstrated effects

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462 on *Bd* growth did not show general patterns with *T. taophora* skin microbiome fungi or other
463 microeukaryotes. As might be expected, bacteria previously found to enhance *Bd* growth were
464 positively associated with the Chytridiomycota, although *Bd* was not present in our 18S dataset.
465 However, *Bd* enhancing bacteria were negatively associated with Ascomycota and
466 Basidiomycota fungi as well as Stramenopiles. Perhaps more critical are the relationships with
467 *Bd* inhibitory bacteria, as these have been proposed for use in probiotic treatments for *Bd*
468 management [14,79]. *Bd* inhibitory bacteria showed significant negative associations with
469 Basidiomycota fungi and other unclassified fungi in the *T. taophora* skin microbiome. *Bd*-
470 inhibitory bacteria were also positively associated with Choanoflagellates, and showed positive
471 although non-significant associations with the Zoopagomycota and Ichthyosporea.
472 These associations demonstrate the importance of understanding potential effects of
473 probiotics on the amphibian skin microbiome and consequently on amphibian health. For
474 example, specific attempts to increase *Bd* inhibiting bacteria and/or reduce *Bd* enhancing bacteria
475 in wild frog populations could reduce fungi in the Dikarya (Ascomycota and Basidiomycota),
476 some of which are known to benefit amphibian health (Kearns et al. 2017), and/or augment
477 poorly studied parasites such as Ichthyosporean protists [80] and Zoopagomycota fungi [81,82]
478 as well as Choanoflagellates that are known to be parasitic in other aquatic ectotherm hosts [83].
479 These hypothetical effects warrant further study, for example through culture-based or *in vivo*
480 challenges between proposed probiotic bacteria and these potentially impacted microeukaryotes.
481 It bears noting that the apparently low susceptibility to *Bd* observed in *Thoropa taophora* [20]
482 may indicate that the results from this study may not apply to more *Bd*-susceptible amphibian
483 species. Nonetheless, our findings demonstrate the importance of understanding cross-domain
484 interactions and microbiome stability as it relates to amphibian health when considering
485 probiotic treatments in wild populations. Evaluation of cross-domain interactions and
486 relationships should be part of the decision-making process in determining whether to employ
487 probiotic conservation strategies.
488
489 *Limitations and future research priorities*
490 Taken together with recent studies [18,19], our results suggest that focusing only on
491 bacteria provides an incomplete picture of the host-associated microbiome. Granted, as in many
492 other amphibian microbiome studies [84] our study presents microbes at a relatively coarse

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phylogenetic resolution. Very large differences in ecology and environmental requirements likely exist between OTUs within higher-order classification levels, and the patterns we detected may change with higher-resolution taxonomic data. With advancing technology allowing for increased sequence length (*e.g.*, third-generation sequencing), more efficient microbiome analysis pipelines (*e.g.,* QIIME2), and higher quality reference sequence databases, future cross-Domain microbiome research at higher taxonomic resolution should be prioritized.

Our results imply that host genetic diversity and MHC IIB genotype play a role in structuring the amphibian skin microbiome. However, we acknowledge that differences in microbiome diversity and structure among site types and MHC IIB genotypes could be due to a number of factors other than or in addition to host genetics. Variation in the microbiome among site types could be explained by differences in environmental filtering in coastal vs. island sites, island isolation favoring longer-dispersing microbes, or alternatively by unexplored host factors (*e.g.*, diet [85]). Additional research is warranted to quantify the relative contributions of host factors, environmental factors, and other variables that contribute to microbiome diversity and structure.

Our network analyses suggest that there may be important interactions between bacteria and microeukaryotes that have been missed by previous microbiome studies focusing on only one microbial Domain or specific microbial interactions. Given the widespread use of bacterial probiotic treatments in humans as well as in domesticated and wild animals [86–88] and the interest in expanding these strategies to wild amphibians [79], future studies should prioritize advancing our understanding of interactions between microbiome bacteria and eukaryotes.

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531

532 **Conflict of Interest**

533 The authors have no conflicts of interest to declare.
534

535 **Author Contributions**

536 **Anat M. Belasen:** Conceptualization (lead), formal analysis (equal), funding acquisition
537 (equal), investigation (equal), methodology (equal), visualization (equal), writing-original draft
538 (lead); **Maria A. Riolo:** Formal analysis (equal), visualization (equal), writing-review & editing
539 (equal); **Molly C. Bletz:** Formal analysis (equal), methodology (equal), validation (equal),
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552

553 **Data Accessibility**

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554 Sequences were deposited in the NCBI Short Read Archive (18S sequences: Accession
555 PRJNA720394; 16S sequences: Accession PRJNA720436). Samples were also registered in
556 SISGEN (#A713DBD).
557

558 **References**

559 1. Lips K *et al.* 2006 Emerging infectious disease and the loss of biodiversity in a
560 Neotropical amphibian community. *Proc. Natl. Acad. Sci.* **103**, 3165–3170.
561 (doi:10.1073/pnas.0506889103)
562 2. Olson DH, Aanensen DM, Ronnenberg KL, Powell CI, Walker SF, Bielby J, Garner TWJ,
563 Weaver G, Fisher MC. 2013 Mapping the Global Emergence of Batrachochytrium
564 dendrobatidis, the Amphibian Chytrid Fungus. *PLoS One* **8**, 747–749.
565 (doi:10.1371/journal.pone.0056802)
566 3. Carvalho T, Becker CG, Toledo LF. 2017 Historical amphibian declines and extinctions in
567 Brazil linked to chytridiomycosis. *Proc. R. Soc. B Biol. Sci.* **284**.
568 (doi:10.1098/rspb.2016.2254)
569 4. Scheele BC *et al.* 2019 Amphibian fungal panzootic causes catastrophic and ongoing loss
570 of biodiversity. *Science (80-.).* **363**, 1459–1463. (doi:10.1126/science.aav0379)
571 5. Muletz C, Caruso NM, Fleischer RC, McDiarmid RW, Lips KR. 2014 Unexpected rarity
572 of the pathogen Batrachochytrium dendrobatidis in Appalachian Plethodon salamanders:
573 1957-2011. *PLoS One* **9**, 103728. (doi:10.1371/journal.pone.0103728)
574 6. Harris RN, James TY, Lauer A, Simon MA, Patel A. 2006 Amphibian pathogen
575 Batrachochytrium dendrobatidis is inhibited by the cutaneous bacteria of amphibian
576 species. *Ecohealth* **3**, 53–56. (doi:10.1007/s10393-005-0009-1)
577 7. Harris RN, Lauer A, Simon MA, Banning JL, Alford RA. 2009 Addition of antifungal
578 skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Dis. Aquat.*
579 *Organ.* **83**, 11–16. (doi:10.3354/dao02004)
580 8. Muletz CR, Myers JM, Domangue RJ, Herrick JB, Harris RN. 2012 Soil bioaugmentation
581 with amphibian cutaneous bacteria protects amphibian hosts from infection by
582 Batrachochytrium dendrobatidis. *Biol. Conserv.* **152**, 119–126.
583 (doi:10.1016/j.biocon.2012.03.022)
584 9. Lam BA, Walke JB, Vredenburg VT, Harris RN. 2010 Proportion of individuals with anti-

Author Manuscript

This article is protected by copyright. All rights reserved

Batrachochytrium dendrobatidis skin bacteria is associated with population persistence in the frog Rana muscosa. *Biol. Conserv.* **143**, 529–531. (doi:10.1016/j.biocon.2009.11.015)

10. Harris RN *et al.* 2009 Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* **3**, 818–824. (doi:10.1038/ismej.2009.27)
11. Vredenburg VT, Briggs CJ, Harris RN. 2011 Host-pathogen dynamics of amphibian chytridiomycosis: The role of the skin microbiome in health and disease. In *Fungal diseases: an emerging threat to human, animal, and plant health.* (eds L Olson, E Choffnes, D Relman, L Pray), pp. 342–355. Washington DC: . National Academy Press.
12. Myers JM, Ramsey JP, Blackman AL, Nichols AE, Minbiole KPC, Harris RN. 2012 Synergistic inhibition of the lethal fungal pathogen Batrachochytrium dendrobatidis: The combined effect of symbiotic bacterial metabolites and antimicrobial peptides of the frog Rana muscosa. *J. Chem. Ecol.* **38**, 958–965. (doi:10.1007/s10886-012-0170-2)
13. Woodhams DC *et al.* 2014 Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS One* **9**. (doi:10.1371/journal.pone.0096375)
14. Bletz MC, Loudon AH, Becker MH, Bell SC, Woodhams DC, Minbiole KPC, Harris RN. 2013 Mitigating amphibian chytridiomycosis with bioaugmentation: Characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* **16**, 807–820. (doi:10.1111/ele.12099)
15. Piovia-Scott J, Rejmanek D, Woodhams DC, Worth SJ, Kenny H, McKenzie V, Lawler SP, Foley JE. 2017 Greater Species Richness of Bacterial Skin Symbionts Better Suppresses the Amphibian Fungal Pathogen Batrachochytrium Dendrobatidis. *Microb. Ecol.* **74**, 217–226. (doi:10.1007/s00248-016-0916-4)
16. Voyles J *et al.* 2018 Shifts in disease dynamics in a tropical amphibian assemblage are not due to pathogen attenuation. *Science (80-.).* **359**, 1517–1519. (doi:10.1126/science.aao4806)
17. Kueneman JG, Woodhams DC, Van Treuren W, Archer HM, Knight R, McKenzie VJ. 2016 Inhibitory bacteria reduce fungi on early life stages of endangered Colorado boreal toads (Anaxyrus boreas). *ISME J.* **10**, 934–944. (doi:10.1038/ismej.2015.168)
18. Kearns PJ, Fischer S, Fernández-Beaskoetxea S, Gabor CR, Bosch J, Bowen JL, Tlusty MF, Woodhams DC. 2017 Fight fungi with fungi: Antifungal properties of the amphibian

Author Manuscript

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616 mycobiome. *Front. Microbiol.* **8**, 1–12. (doi:10.3389/fmicb.2017.02494)
617 19. Kueneman JG, Weiss S, McKenzie VJ. 2017 Composition of micro-eukaryotes on the
618 skin of the cascades frog (Rana cascadae) and patterns of correlation between skin
619 microbes and Batrachochytrium dendrobatidis. *Front. Microbiol.* **8**, 1–10.
620 (doi:10.3389/fmicb.2017.02350)
621 20. Belasen AM, Bletz MC, Leite D da S, Toledo LF, James TY. 2019 Long-Term Habitat
622 Fragmentation Is Associated With Reduced MHC IIB Diversity and Increased Infections
623 in Amphibian Hosts. *Front. Ecol. Evol.* **6**, 1–12. (doi:10.3389/fevo.2018.00236)
624 21. Gao F-K, Dai C-C, Liu X-Z. 2010 Mechanisms of fungal endophytes in plant protection
625 against pathogens. *African J. Microbiol. Res.* **4**, 1346–1351.
626 22. Newsham KK, Fitter AH, Watkinson AR. 1995 Arbuscular Mycorrhiza Protect an Annual
627 Grass from Root Pathogenic Fungi in the Field. *J. Ecol.* **83**, 991. (doi:10.2307/2261180)
628 23. Gleason FH, Carney LT, Lilje O, Glockling SL. 2012 Ecological potentials of species of
629 Rozella (Cryptomycota). *Fungal Ecol.* **5**, 651–656. (doi:10.1016/j.funeco.2012.05.003)
630 24. Hoffmann AR *et al.* 2014 The skin microbiome in healthy and allergic dogs. *PLoS One* **9**.
631 (doi:10.1371/journal.pone.003197)
632 25. Holler E *et al.* 2014 Metagenomic Analysis of the Stool Microbiome in Patients Receiving
633 Allogeneic Stem Cell Transplantation: Loss of Diversity Is Associated with Use of
634 Systemic Antibiotics and More Pronounced in Gastrointestinal Graft-versus-Host Disease.
635 *Biol. Blood Marrow Transplant.* **20**, 640–645. (doi:10.1016/j.bbmt.2014.01.030)
636 26. Graham AL. 2008 Ecological rules governing helminth microparasite coinfection. *Proc.*
637 *Natl. Acad. Sci.* **105**, 566–570. (doi:10.1073/pnas.0707221105)
638 27. Benson AK *et al.* 2010 Individuality in gut microbiota composition is a complex
639 polygenic trait shaped by multiple environmental and host genetic factors. *Proc. Natl.*
640 *Acad. Sci.* **107**, 18933–18938. (doi:10.1073/pnas.1007028107)
641 28. Blekhman R *et al.* 2015 Host genetic variation impacts microbiome composition across
642 human body sites. *Genome Biol.* **16**. (doi:10.1186/s13059-015-0759-1)
643 29. Marietta E, Rishi A, Taneja V. 2015 Immunogenetic control of the intestinal microbiota.
644 *Immunology* **145**, 313–322. (doi:10.1111/imm.12474)
645 30. Kueneman JG, Parfrey LW, Woodhams DC, Archer HM, Knight R, McKenzie VJ. 2014
646 The amphibian skin-associated microbiome across species, space and life history stages.

Author Manuscript

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647 *Mol. Ecol.* **23**, 1238–1250. (doi:10.1111/mec.12510)
648 31. Walke JB, Becker MH, Loftus SC, House LL, Cormier G, Jensen R V, Belden LK. 2014
649 Amphibian skin may select for rare environmental microbes. *ISME J.* **8**, 1–11.
650 (doi:10.1038/ismej.2014.77)
651 32. Griffiths SM, Harrison XA, Weldon C, Wood MD, Pretorius A, Hopkins K, Fox G,
652 Preziosi RF, Antwis RE. 2018 Genetic variability and ontogeny predict microbiome
653 structure in a disease-challenged montane amphibian. *ISME J.* , 1–12.
654 (doi:10.1038/s41396-018-0167-0)
655 33. Barribeau SM, Villinger J, Waldman B. 2012 Ecological immunogenetics of life-history
656 traits in a model amphibian. *Biol. Lett.* **8**, 405–407. (doi:Doi 10.1098/Rsbl.2011.0845)
657 34. Allentoft ME, O'Brien J. 2010 Global amphibian declines, loss of genetic diversity and
658 fitness: A review. *Diversity* **2**, 47–71. (doi:10.3390/d2010047)
659 35. Rebollar EA, Simonetti SJ, Shoemaker WR, Harris RN. 2016 Direct and indirect
660 horizontal transmission of the antifungal probiotic bacterium Janthinobacterium lividum
661 on green frog (Lithobates clamitans) tadpoles. *Appl. Environ. Microbiol.* **82**, 2457–2466.
662 (doi:10.1128/AEM.04147-15)
663 36. Adair KL, Douglas AE. 2017 Making a microbiome: the many determinants of host-
664 associated microbial community composition. *Curr. Opin. Microbiol.* **35**, 23–29.
665 (doi:10.1016/j.mib.2016.11.002)
666 37. Tilman D, May RM, Lehman CL, Nowak MA. 1994 Habitat destruction and the
667 extinction debt. *Nature* **371**, 65–66. (doi:10.1038/371065a0)
668 38. Suguio K, Angulo RJ, Carvalho AM, Corrêa ICS, Tomazeli LJ, Willwock JA, Vital. H.
669 2005 Paleoníveis do mar e paleolinhas da costa. In *Quaternário do Brasil*, p. 378.
670 39. Bell RC, Brasileiro CA, Haddad CFB, Zamudio KR. 2012 Evolutionary history of Scinax
671 treefrogs on land-bridge islands in south-eastern Brazil. *J. Biogeogr.* **39**, 1733–1742.
672 (doi:10.1111/j.1365-2699.2012.02708.x)
673 40. Duryea MC, Zamudio KR, Brasileiro CA. 2015 Vicariance and marine migration in
674 continental island populations of a frog endemic to the Atlantic Coastal forest. *Heredity*
675 *(Edinb).* **115**, 225–234. (doi:10.1038/hdy.2015.31)
676 41. Duryea MC, Zamudio KR, Brasileiro CA. 2008 Characterization of microsatellite markers
677 for Thoropa taophora (Anura, Cycloramphidae), a frog endemic to the Brazilian Atlantic

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678 Rain Forest. *Mol. Ecol. Resour.* **8**, 663–665. (doi:10.1111/j.1471-8286.2007.02039.x)
679 42. Hyatt AD *et al.* 2007 Diagnostic assays and sampling protocols for the detection of
680 Batrachochytrium dendrobatidis. *Dis. Aquat. Organ.* **73**, 175–192.
681 (doi:10.3354/dao073175)
682 43. Vences M *et al.* 2016 Gut bacterial communities across tadpole ecomorphs in two diverse
683 tropical anuran faunas. *Sci. Nat.* **103**. (doi:10.1007/s00114-016-1348-1)
684 44. Stoeck T, Bass D, Nebel M, Christen R, Jones MDM, Breiner HW, Richards TA. 2010
685 Multiple marker parallel tag environmental DNA sequencing reveals a highly complex
686 eukaryotic community in marine anoxic water. *Mol. Ecol.* **19**, 21–31. (doi:10.1111/j.1365-
687 294X.2009.04480.x)
688 45. Caporaso JG *et al.* 2010 QIIME allows analysis of high-throughput community
689 sequencing data. *Nat. Methods* **7**. (doi:10.1038/nmeth.f.303)
690 46. Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA,
691 Caporaso JG. 2013 Quality-filtering vastly improves diversity estimates from Illumina
692 amplicon sequencing. *Nat. Methods* **10**, 57–59. (doi:10.1038/nmeth.2276)
693 47. Edgar RC. 2016 UCHIME2: improved chimera prediction for amplicon sequencing.
694 (doi:10.1101/074252)
695 48. Bletz MC *et al.* 2016 Amphibian gut microbiota shifts differentially in community
696 structure but converges on habitat-specific predicted functions. *Nat. Commun.* **7**, 1–12.
697 (doi:10.1038/ncomms13699)
698 49. Bletz MC, Vences M, Sabino-Pinto J, Taguchi Y, Shimizu N, Nishikawa K, Kurabayashi
699 A. 2017 Cutaneous microbiota of the Japanese giant salamander (Andrias japonicus), a
700 representative of an ancient amphibian clade. *Hydrobiologia* **795**, 153–167.
701 (doi:10.1007/s10750-017-3126-2)
702 50. Woodhams DC *et al.* 2015 Antifungal isolates database of amphibian skin-associated
703 bacteria and function against emerging fungal pathogens. *Ecology* **96**, 595–595.
704 (doi:10.1890/14-1837.1)
705 51. R Core Team. 2018 R: A language and environment for statistical computing. *R Found.*
706 *Stat. Comput. Vienna, Austria.*
707 52. Song Z, Schlatter D, Gohl DM, Kinkel LL. 2018 Run-to-run sequencing variation can
708 introduce taxon-specific bias in the evaluation of fungal microbiomes. *Phytobiomes J.* **2**,

Author Manuscript

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709 165–170. (doi:10.1094/PBIOMES-09-17-0041-R)

710 53. Kembel SW, Wu M, Eisen JA, Green JL. 2012 Incorporating 16S Gene Copy Number
711 Information Improves Estimates of Microbial Diversity and Abundance. *PLoS Comput.*
712 *Biol.* **8**, 16–18. (doi:10.1371/journal.pcbi.1002743)

713 54. Větrovský T, Baldrian P. 2013 The Variability of the 16S rRNA Gene in Bacterial
714 Genomes and Its Consequences for Bacterial Community Analyses. *PLoS One* **8**, 1–10.
715 (doi:10.1371/journal.pone.0057923)

716 55. Pinto AJ, Raskin L. 2012 PCR biases distort bacterial and archaeal community structure in
717 pyrosequencing datasets. *PLoS One* **7**. (doi:10.1371/journal.pone.0043093)

718 56. Dray S, Dufour AB. 2007 The ade4 package: implementing the duality diagram for
719 ecologists. *J. Stat. Softw.* **22**, 1–20.

720 57. Chessel D, Dufour AB, Thioulouse J. 2004 The ade4 package-I- One-table methods. *R*
721 *News* **4**, 5–10.

722 58. Dray S, Dufour AB, Chessel D. 2007 The ade4 package-II: Two-table and K-table
723 methods. *R News* **7**, 47–52.

724 59. Hunter JD. 2007 Matplotlib: A 2D Graphics Environment. *Comput. Sci. Eng.* **9**, 90–95.

725 60. van Rossum G. 1995 Python Tutorial, Technical Report CS-R9526.

726 61. Petersen J. 1891 Die Theorie der regulären graphs. *Acta Math.* **15**, 193–220.
727 (doi:10.1007/BF02392606)

728 62. Besag J, Clifford P. 1989 Generalized Monte Carlo significance tests. *Biometrika* **76**, 633–
729 642. (doi:10.1093/biomet/76.4.633)

730 63. Fosdick BK, Larremore DB, Nishimura J, Ugander J. 2018 Configuring Random Graph
731 Models with Fixed Degree Sequences. *SIAM Rev.* **60**, 315–355.
732 (doi:10.1137/16M1087175)

733 64. Benjamini Y, Hochberg Y, Benjamini, Yoav HY. 1995 Benjamini and Y FDR.pdf. *J. R.*
734 *Stat. Soc. Ser. B*. **57**, 289–300. (doi:10.2307/2346101)

735 65. McKenzie VJ, Bowers RM, Fierer N, Knight R, Lauber CL. 2012 Co-habiting amphibian
736 species harbor unique skin bacterial communities in wild populations. *ISME J.* **6**, 588–
737 596. (doi:10.1038/ismej.2011.129)

738 66. Abarca JG, Vargas G, Zuniga I, Whitfield SM, Woodhams DC, Kerby J, McKenzie VJ,
739 Murillo-Cruz C, Pinto-Tomás AA. 2018 Assessment of bacterial communities associated

Author Manuscript

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740 with the skin of Costa Rican amphibians at La Selva Biological Station. *Front. Microbiol.*
741 **9**, 1–12. (doi:10.3389/fmicb.2018.02001)
742 67. Belden LK *et al.* 2015 Panamanian frog species host unique skin bacterial communities.
743 *Front. Microbiol.* **6**, 1–21. (doi:10.3389/fmicb.2015.01171)
744 68. Bletz MC, Archer H, Harris RN, McKenzie VJ, Rabemananjara FCE, Rakotoarison A,
745 Vences M. 2017 Host ecology rather than host phylogeny drives amphibian skin microbial
746 community structure in the biodiversity hotspot of Madagascar. *Front. Microbiol.* **8**, 1–14.
747 (doi:10.3389/fmicb.2017.01530)
748 69. Varela BJ, Lesbarrères D, Ibáñez R, Green DM. 2018 Environmental and host effects on
749 skin bacterial community composition in Panamanian frogs. *Front. Microbiol.* **9**, 1–13.
750 (doi:10.3389/fmicb.2018.00298)
751 70. Kueneman JG *et al.* 2019 Community richness of amphibian skin bacteria correlates with
752 bioclimate at the global scale. *Nat. Ecol. Evol.* **3**, 381–389. (doi:10.1038/s41559-019-
753 0798-1)
754 71. Hill WA, Newman SJ, Craig L, Carter C, Czarra J, Brown JP. 2010 Diagnosis of
755 Aeromonas hydrophila, Mycobacterium species, and Batrachochytrium dendrobatidis in
756 an African Clawed Frog (Xenopus laevis). *J. Am. Assoc. Lab. Anim. Sci.* **49**, 215–220.
757 72. Becker MH *et al.* 2015 Phylogenetic distribution of symbiotic bacteria from Panamanian
758 amphibians that inhibit growth of the lethal fungal pathogen Batrachochytrium
759 dendrobatidis. *Mol. Ecol.* **24**, 1628–1641. (doi:10.1111/mec.13135)
760 73. Brucker RM, Baylor CM, Walters RL, Lauer A, Harris RN, Minbiole KPC. 2008 The
761 identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by
762 cutaneous bacteria of the salamander plethodon cinereus. *J. Chem. Ecol.* **34**, 39–43.
763 (doi:10.1007/s10886-007-9352-8)
764 74. Amend AS, Seifert KA, Bruns TD. 2010 Quantifying microbial communities with 454
765 pyrosequencing: Does read abundance count? *Mol. Ecol.* **19**, 5555–5565.
766 (doi:10.1111/j.1365-294X.2010.04898.x)
767 75. Hernández-Gómez O, Hoverman JT, Williams RN. 2017 Cutaneous microbial community
768 variation across populations of eastern hellbenders (Cryptobranchus alleganiensis
769 alleganiensis). *Front. Microbiol.* **8**, 1–16. (doi:10.3389/fmicb.2017.01379)
770 76. Hernández-Gómez O, Briggler JT, Williams RN. 2018 Influence of immunogenetics, sex

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771 and body condition on the cutaneous microbial communities of two giant salamanders.
772 *Mol. Ecol.* **27**, 1915–1929. (doi:10.1111/mec.14500)
773 77. Deveau A *et al.* 2018 Bacterial-fungal interactions: Ecology, mechanisms and challenges.
774 *FEMS Microbiol. Rev.* **42**, 335–352. (doi:10.1093/femsre/fuy008)
775 78. Frey-Klett P, Garbaye J. 2005 Mycorrhiza Helper Bacteria: A Promising Model for the
776 Genomic Analysis of Fungal- Bacterial Interactions. *New Phytol.* **168**, 4–8.
777 79. Walke JB, Belden LK. 2016 Harnessing the Microbiome to Prevent Fungal Infections:
778 Lessons from Amphibians. *PLoS Pathog.* **12**, 6–11. (doi:10.1371/journal.ppat.1005796)
779 80. Rowley JJL, Gleason FH, Andreou D, Marshall WL, Lilje O, Gozlan R. 2013 Impacts of
780 mesomycetozoean parasites on amphibian and freshwater fish populations. *Fungal Biol.*
781 *Rev.* **27**, 100–111. (doi:10.1016/j.fbr.2013.09.002)
782 81. Badali H *et al.* 2010 Rhinocladiella aquaspersa, proven agent of verrucous skin infection
783 and a novel type of chromoblastomycosis. *Med. Mycol.* **48**, 696–703.
784 (doi:10.3109/13693780903471073)
785 82. Seyedmousavi S, Guillot J, Tolooe A, Verweij PE. 2015 Neglected fungal zoonoses:
786 hidden threats to man and animals. *Clin. Microbiol. Infect.* **21**, 416–425.
787 (doi:10.1016/j.cmi.2015.02.031)
788 83. Kerk D, Gee A, Standish M, Wainwright PO, Drum AS, Elston RA, Sogin ML. 1995 The
789 rosette agent of chinook salmon (Oncorhynchus tshawytscha) is closely related to
790 choanoflagellates, as determined by the phylogenetic analyses of its small ribosomal
791 subunit RNA. *Mar. Biol.* **122**, 187–192. (doi:10.1007/BF00348931)
792 84. McKenzie VJ, Bowers RM, Fierer N, Knight R, Lauber CL. 2012 Co-habiting amphibian
793 species harbor unique skin bacterial communities in wild populations. *ISME J.* **6**, 588–
794 596. (doi:10.1038/ismej.2011.129)
795 85. Antwis RE, Haworth RL, Engelmoer DJP, Ogilvy V, Fidgett AL, Preziosi RF. 2014 Ex
796 situ diet influences the bacterial community associated with the skin of red-eyed tree frogs
797 (agalychnis callidryas). *PLoS One* **9**, 1–8. (doi:10.1371/journal.pone.0085563)
798 86. Gram L, Melchiorsen J, Spanggaard B, Huber I, Al GET, Icrobiol APPLENM. 1999 AH2
799 , a Possible Probiotic Treatment of Fish. *Appl. Environ. Microbiol.* **65**, 969–973.
800 87. Ghadban GS. 2002 Probiotics in broiler production - A review. *Arch. fur Geflugelkd.* **66**,
801 49–58.

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802 88. Cheng TL *et al.* 2017 Efficacy of a probiotic bacterium to treat bats affected by the
803 disease white-nose syndrome. *J. Appl. Ecol.* **54**, 701–708. (doi:10.1111/1365-2664.12757)
804

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Table 1: Sampling site data. Sample size is the number of frogs collected at each site. MHC IIB heterozygosity is the observed heterozygosity, or number of heterozygotes over the total individuals genotyped from each population.

Site name	Site code	Site type	Latitude	Longitude	Sample size	MHC IIB heterozygosity, H_O	MHC IIB Allelic Richness, N_A
As Ilhas	AI	Island	-23.789276	-45.711507	4	0	1
Couve Sul	CS	Island	-23.800899	-45.721672	7	0.43	2
Couves Norte	CN	Island	-23.422075	-44.854066	30	0.43	2
Gatos	GA	Island	-23.805592	-45.670011	3	0	1
Porcos Pequena	PP	Island	-23.377864	-44.904266	20	0	1
Prumirim	PR	Island	-23.384791	-44.945678	22	0.09	4
Tamandua	TA	Island	-23.597168	-45.288857	25	0	1
Barra do Una	BU	Coastal	-23.761536	-45.770697	20	0	2
Sununga	SU	Coastal	-23.508867	-45.133827	20	0.7	11
Toque Toque	TT	Coastal	-23.835912	-45.509922	24	0.51	10

Site locations are shown in Figure 1.

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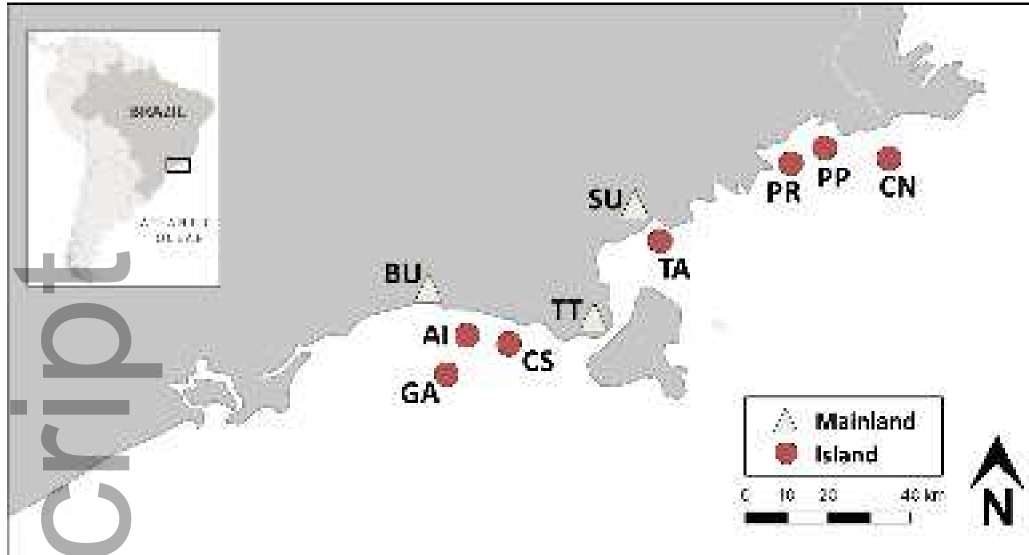

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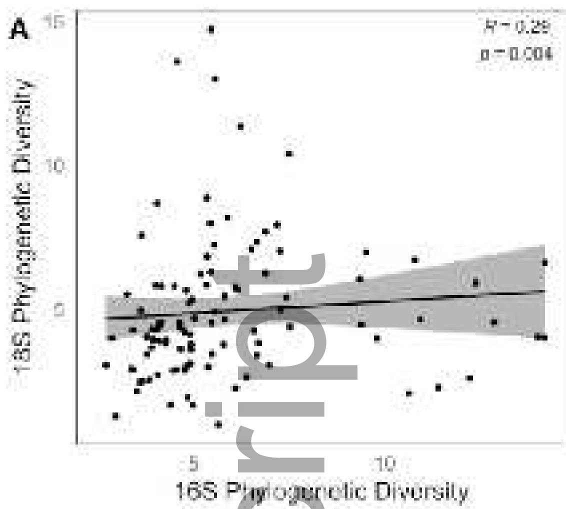

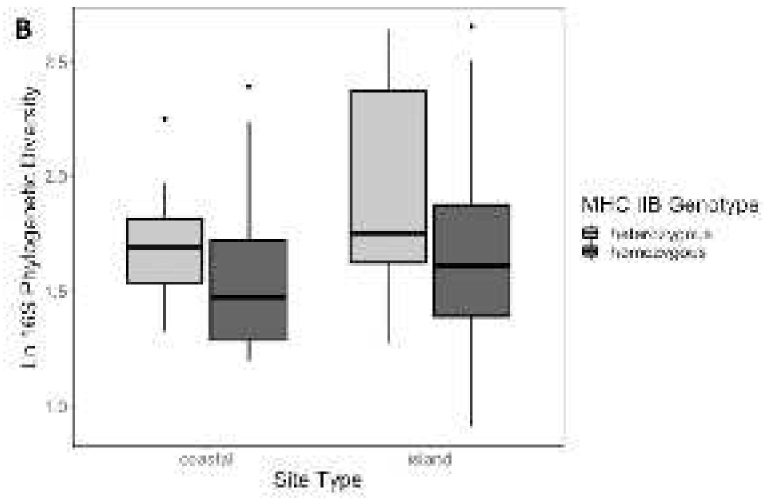

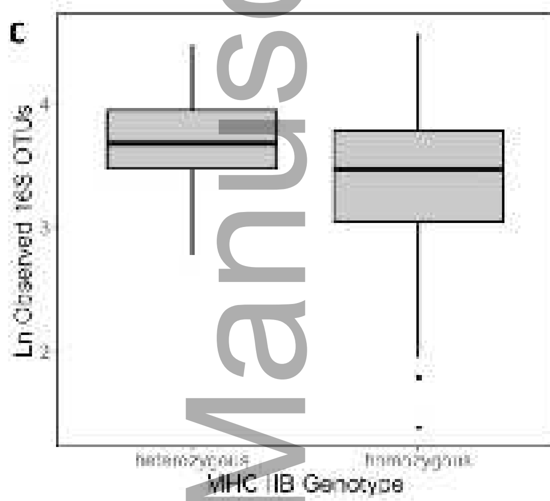

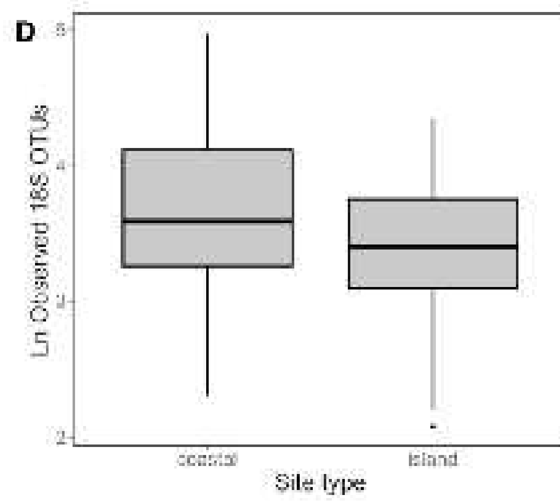

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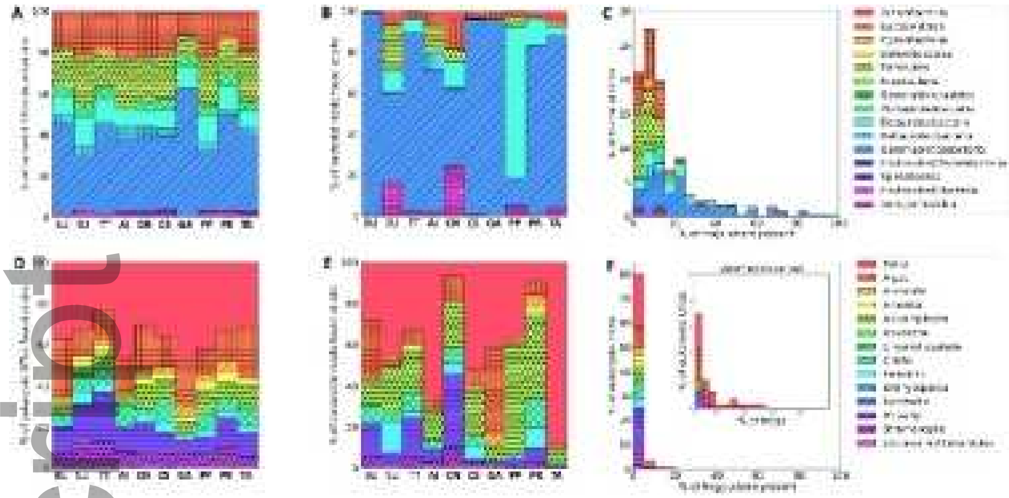

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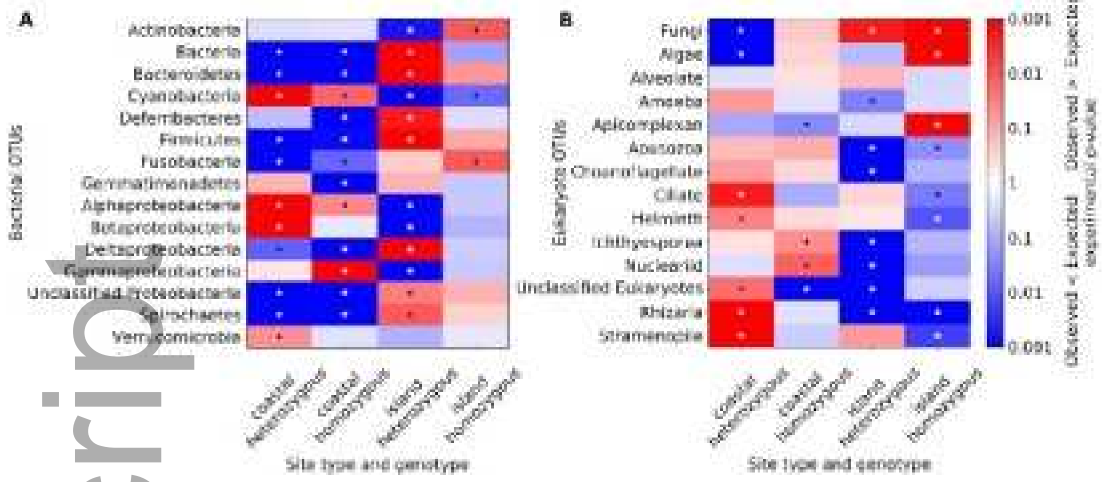

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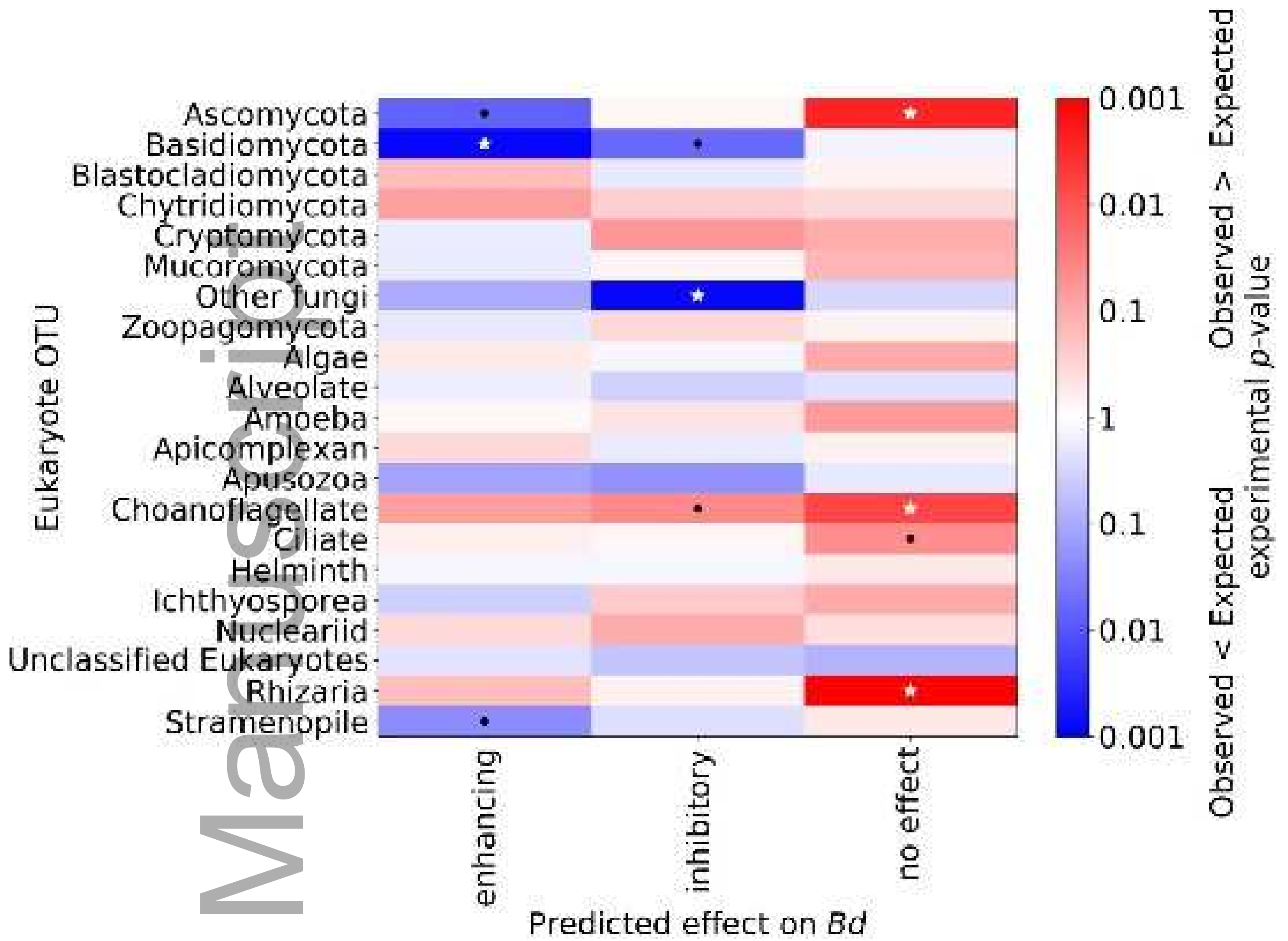

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