The effect of captivity on the skin microbial symbionts in three *Atelopus* species from the lowlands of Colombia and Ecuador

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Many amphibian species are at risk of extinction in their natural habitats due to the presence of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). For the most highly endangered species, captive assurance colonies have been established as an emergency measure to avoid extinction. Experimental research has suggested that symbiotic microorganisms in the skin of amphibians play a key role against *Bd*. While previous studies have addressed the effects of captivity on the cutaneous bacterial community, it remains poorly studied whether and how captive conditions affect the proportion of beneficial bacteria or their anti-*Bd* performance on amphibian hosts. In this study we sampled three amphibian species of the highly threatened genus, *Atelopus*, that remain in the wild but are also part of *ex situ* breeding programs in Colombia and Ecuador. Our goals were to (1) estimate the diversity of culturable bacterial assemblages in these three species of *Atelopus*, (2) describe the effect of captivity on the composition of skin microbiota, and (3) examine how captivity affects the bacterial ability to inhibit *Bd* growth. Using challenge assays we tested each bacterial isolate against *Bd*, and through sequencing of the 16S rRNA gene, we identified species from thirteen genera of bacteria that inhibited *Bd* growth. Surprisingly, we did not detect a reduction in skin bacteria diversity in captive frogs. Moreover, we found that frogs in captivity still harbor bacteria with anti-*Bd* activity. Although the scope of our study is limited to a few species and to the culturable portion of the bacterial community, our results indicate that captive programs do not necessarily change bacterial communities of the toad skins in a way that impedes the control of *Bd* in case of an eventual reintroduction.

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20 **Abstract**

22 Many amphibian species are at risk of extinction in their natural habitats due to the presence of

23 the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). For the most highly endangered

24 species, captive assurance colonies have been established as an emergency measure to avoid

25 extinction. Experimental research has suggested that symbiotic microorganisms in the skin of

26 amphibians play a key role against *Bd*. While previous studies have addressed the effects of

27 captivity on the cutaneous bacterial community, it remains poorly studied whether and how

28 captive conditions affect the proportion of beneficial bacteria or their anti-*Bd* performance on

29 amphibian hosts. In this study we sampled three amphibian species of the highly threatened

30 genus, *Atelopus*, that remain in the wild but are also part of *ex situ* breeding programs in

31 Colombia and Ecuador. Our goals were to (1) estimate the diversity of culturable bacterial

32 assemblages in these three species of *Atelopus*, (2) describe the effect of captivity on the

33 composition of skin microbiota, and (3) examine how captivity affects the bacterial ability to

34 inhibit *Bd* growth. Using challenge assays we tested each bacterial isolate against *Bd*, and

35 through sequencing of the 16S rRNA gene, we identified species from thirteen genera of bacteria

36 that inhibited *Bd* growth. Surprisingly, we did not detect a reduction in skin bacteria diversity in

37 captive frogs. Moreover, we found that frogs in captivity still harbor bacteria with anti-*Bd*

38 activity. Although the scope of our study is limited to a few species and to the culturable portion

39 of the bacterial community, our results indicate that captive programs do not necessarily change

40 bacterial communities of the toad skins in a way that impedes the control of *Bd* in case of an

41 eventual reintroduction.

42

43

Introduction

45 Global amphibian declines have been attributed to a variety of factors including habitat

46 destruction, contamination, UV-B radiation, climate change, overexploitation, and infectious

47 diseases (Alford & Richards, 1999; Collins & Crump, 2009; Heard et al., 2013). Diseases caused

48 by microparasites have been linked to massive mortality events and extinctions, and the

49 causative agents can be viral, bacterial, or caused by protists and fungi (Latney & Klaphake,

50 2013; Chambouvet et al., 2015). Emerging diseases caused by fungi have become more

51 problematic and the impact on the affected groups are much more alarming (Wake and

52 Vredenburg, 2008; Fisher et al., 2012). *Batrachochytrium dendrobatidis* (*Bd*) (Longcore, Pessier

53 & Nichols, 1999), and *B. salamandrivorans* (*Bsal*) (Martel et al., 2013) are highly pathogenic

54 fungi, recognized as the etiological agents of the amphibian infectious disease known as

55 chytridiomycosis. *Bsal* has been detected only in urodels (salamanders and newts) (Martel et al.,

56 2013; 2014), while *Bd* is considered a generalist pathogen found in a wide variety of amphibian

57 species across the three orders (i.e., Anura, Urodela and Gymnophiona). *Bd* has been detected in

58 at least 520 amphibian species in 56 countries (Berger et al., 2016) causing population declines

59 in at least 200 species across five continents (Skerratt et al., 2007; Fisher, Garner & Walker,

60 2009).

61

62 Conservation strategies focused on ameliorating the lethal effects of the fungal pathogen have

63 been mainly restricted to establishing disease-free *ex situ* colonies, with the aim of ensuring the

64 survival of susceptible species (Becker et al., 2014; Tapley et al., 2015). Captive programs are a

65 short-term intervention to prevent extinction where the final goal is to reintroduce the animals to

66   their native habitat (Mendelson et al., 2006). Although, a strategy that ensures host survival

67   despite *Bd* presence is still needed. Meanwhile*, ex situ* programs may be the best option for many

68   threatened amphibians occurring in complex environments, where *in situ* strategies are difficult

69   to apply (Bosch et al., 2015). The conditions under which amphibians are maintained in captivity

70   may disturb their associated symbiotic microbes (Kueneman et al., 2016; Loudon et al. 2014),

71   which would affect health status (Li et al., 2008; Becker et al., 2015) and survival rates if

72   released into the wild (Redford et al., 2012; Michaels, Downie & Campbell-Palmer, 2014b).

73

74   Cutaneous microbes can inhibit or delay the growth of *Bd* (Harris et al., 2006; 2009; Woodhams

75   et al., 2007), and therapies where beneficial microbes are augmented in the host skin have been

76   considered as an option to treat *Bd*-susceptible species *in situ* (Woodhams et al., 2011; Bletz et

77   al., 2013; Woodhams et al., 2016). However, new approaches should take into account the

78   complex interactions among the host, its symbiotic bacteria and the fungal pathogen, without

79   neglecting the role of the environmental context on these interactions (McKenzie et al., 2012;

80   Kueneman et al., 2014; Bosch et al., 2015). Captivity imposes a different environment that might

81   affect the microbial composition, and thus, the response of the host to the pathogen. Various

82   studies have suggested that skin associated microbes of individuals in captivity differ from those

83   in the wild (Becker et al., 2014, Kueneman et al., 2016, Sabino-Pinto et al., 2016). In the case of

84   the red-eyed tree frog (*Agalychnis callidryas*)*,* community composition, species richness, and

85   abundance of bacterial groups seem to be influenced mainly by the availability of carotenoids in

86   their diet (Antwis et al., 2014), and by the cover provided in the enclosures (Michaels, Antwis &

87   Preziosi, 2014a). However, other factors including humidity, temperature, pH and disinfection

88   methods affect the presence of certain bacterial species and could facilitate or impede

89   colonization and establishment in the host skin (Mendoza et al., 2012). Some studies have

90   demonstrated that OTU (Operational Taxonomic Units) richness and phylogenetic diversity are

91   significantly higher in captive animals (i.e., in *Atelopus zeteki,* Becker et al., 2014), while other

92   studies find the opposite, where wild animals harbor a higher bacterial diversity (i.e. *Cynops*

93   *pyrrhogaster,* Sabino-Pinto et al., 2016 and, *Anaxyrus boreas,* Kueneman et al., 2016).

94

95   Although changes in the bacterial assemblage composition due to captivity seem to vary

96   depending on the species, understanding how changes in the bacterial community structure affect

97   the host response to *Bd* infection may be key to successful *ex situ* programs. For example,

98   Becker et al. (2015) showed that survival rates after exposure to *Bd* seem to be associated with

99   the initial composition of the skin bacterial community, so different initial communities result in

100   different outcomes for the host. Even though scientists have provided important insights on how

101   different factors affect microbial assemblages in captive conditions (Antwis et al., 2014;

102   Michaels, Antwis & Preziosi, 2014a; Michaels, Downie & Campbell-Palmer, 2014b), additional

103   research is required to determine the impact of captivity on the proportion of bacteria with anti-

104   *Bd* properties for specific amphibian species. Knowing how captive conditions affect the

105   proportion and performance of bacteria with antifungal capacities will allow us to more

106   accurately predict the fate of species considered for reintroduction into the wild.

107

108   Harlequin toads from the genus *Atelopus* are considered one of the most threatened groups of

109   amphibians worldwide, with at least 71 % of the species listed as critically endangered according

110   to the IUCN (2016). Although many species have been declining, some species from the

111   lowlands still persist in their natural habitats (Flechas, Vredenburg & Amézquita, 2015). Other

112   species, such as *A. elegans* from Ecuador, has only been found in one of its historical collection

113   localities. Given the crisis of wild amphibian populations and the highly susceptibility of

114   *Atelopus* to the fungal disease, conservation actions have been focused on keeping assurance

115   colonies in order to protect these species. Conservation initiatives are committed to protecting

116   those species facing the highest risk of extinction, and have included at least 15 species of the

117   genus *Atelopus* in rescue programs (http://progress.amphibianark.org/model-programs). In this

118   study, we evaluated the effect of *ex situ* conditions on bacteria with anti-*Bd* activity. To

119   accomplish this, we performed antagonism assays to determine which bacterial species can

120   inhibit *Bd* growth. Then we compared the activity between bacteria isolated from individuals in

121   the wild with those maintained in captivity. We use culture-dependent methods to compare the

122   skin bacterial community in three *Atelopus* species that persist in the wild but are also part of an

123   *ex situ* breeding program. Our main goal was to evaluate the effect of captivity on the

124   composition of the skin microbial community, focusing mainly in those that exhibit anti-*Bd*

125   properties. Since captive environments have been described as less heterogeneous and less

126   diverse compared with natural habitats, we hypothesized that animals in the wild would support

127   a more diverse bacterial community and therefore a higher capacity to inhibit *Bd* growth,

128   increasing chances of host survival.

129

130   **Materials & Methods**

131

132   Research permits to conduct this study were provided by the Colombian National Parks

133   Authority, the Ministerio de Ambiente de Colombia, and the Ministerio del Ambiente Ecuador

134   (MAE) under permits DTSO 019-09, DTSO 001-09, Nº 10-07032012, 001-11 IC-FAU-

135   DNB/MA and 11-2012-FAU-DPAP-MA.

136

137   *Study species*

138   We studied three *Atelopus* species from the lowlands, two occurring in the Pacific coastal forests

139   of Colombia and one in Ecuador (Fig. 1). We took skin bacterial samples of *A. spurrelli*, in the

140   municipality of Arusí (5.57ºN, 77.50ºW; 90 m), *A.* aff. *limosus*, a species occurring near

141   Capurganá (8.60°N, 77.33°W; 150 m) near the border between Colombia and Panama, and *A.*

142   *elegans* from the Esmeraldas Province in northwestern Ecuador (1.04ºN, 78.62ºW; 265 m). In

143   addition, we took skin swabs from captive *A. elegans, A.* aff. *limosus* and *A. spurrelli* from the *ex*

144   *situ* programs in the Cali Zoo (Cali, Colombia) and the "Balsa de los Sapos" Conservation

145   Initiative at the Pontificia Universidad Católica del Ecuador (Quito, Ecuador). Individuals in

146   captivity came from the same populations where wild samples were collected (Table 1).

147

148   **Ex situ conditions**

149   *Atelopus spurrelli* and *A.* aff. *limosus* were brought to the Cali Zoo facilities and kept isolated

150   during quarantine (60 days). Then individuals were transferred to the enclosures (1 m x 0.5 m x

151   0.5 m) built using 6 mm-thick glass. To better simulate natural conditions, we created a 10 cm-

152   depth pool that occupied a fifth of the tank's base. Each terrarium was connected to an external

153   canister filter AE 306 Resun® (flow: 700L/H, power: 12 watts). The filters were placed below

154   each terrarium and connected with ½" tubing. Natural plants were placed in the tank to provide

155   shelter, sleeping sites and visual barriers for the frogs, as well as to maintain high humidity, good

156   water quality and low levels of nitrogenous waste. Bromeliads, aroids, mosses, orchids and

157   calatheas were preferred because they tolerate high humidity levels. All individuals were fed five

158   times a week with domestic crickets and fruit flies enriched with vitamin-mineral supplements.

159   Because our study species occur in the lowlands of Colombia, we kept air temperature within

160   enclosures between 24 – 30 °C and water temperature at 20.5 – 22 °C.

161

162   Individuals of *A. elegans* at "Balsa de los Sapos" Conservation Initiative were maintained

163   isolated in independent containers during quarantine (60 days). Afterward, toads were transferred

164   to plastic enclosures (30 cm x 20 cm x 20 cm) in which they were kept individually. Each

165   terrarium contained natural plants, leaf litter, and a coconut bark to provide shelter and sleeping

166   sites for the toads. Each container had 3 cm of water under false floor to maintain high levels of

167   relative humidity. Clean water simulating artificial rain fell four times a day into each container

168   during 5 minutes from a purification system with four filters: polyester film, UV, activated

169   charcoal, and fabric filtration. All toads were fed three times a week with crickets that were

170   sprinkled with vitamins and calcium supplement (Repashi Superfoods). Room temperature

171   ranged between 19.6 – 22.9 °C.

172

173   *Bacterial isolation*

174   In order to determine the effect of captive conditions on the composition of cutaneous bacteria,

175   we took skin bacterial samples from captive animals: four *A. spurrelli*, seven *A.* aff. *limosus* and

176   ten individuals of *A. elegans*. All bacterial swab samples from captive animals were collected in

177   2012 (Table 1). To compare bacterial communities of captive and wild frogs, samples from wild

178   animals were taken from five individuals of *A. elegans* from Esmeraldas (Ecuador). For wild

179   individuals of *A. spurrelli* and *A.* aff. *limosus* we used the published data from Flechas et al.

180   (2012).

181

182   Individuals were handled with new nitrile gloves and rinsed with 20 mL of sterile water to

183   remove transient cutaneous microbiota. Each animal was sampled by running a sterile synthetic

184   rayon swab (Medical Wire Equipment MWE 100) on their left, right of their body and ventral

185   surfaces, hindlimbs, and interdigital membranes for a total of 50 strokes. Swabs were stored in 2

186   mL cryovials containing 1 mL DS solution, i.e., a weak salt solution resembling pond water

187   (Boyle et al., 2003). Samples were refrigerated within 24 h and processed within 48 h after

188   sampling. To isolate pure colonies, we followed the protocol described previously by Flechas et

189   al. (2012). Each isolate was cryopreserved in nutritive broth with 30% glycerol at -80ºC.

190

191   *Growth inhibition assays*

192   To determine the ability of each isolate to inhibit *Bd* growth we performed antagonism assays as

193   previously published (Harris et al., 2006; Lam et al., 2010; Flechas et al., 2012). Assays were

194   conducted using two *Bd* strains: JEL423 (provided by J. E. Longcore, University of Maine,

195   Orono, USA) and EV001 (Flechas et al., 2013). Bacterial isolates from captive animals of the

196   three *Atelopus* species and from wild *A. elegans* were challenged against both *Bd* strains in

197   duplicate. Bacterial strains isolated from wild Colombian *Atelopus* were only tested against the

198   strain JEL 423 since the Colombian strain was not available before 2012. Query bacteria that

199   produced a clear halo were considered as inhibitory (see Fig 1 in Flechas et al., 2012). Isolates

200   that showed signs of growth inhibition were photographed under similar resolution and lighting

201   conditions in a dark background in order to estimate antifungal ability.

202

203 Sections of approximately 3.0 cm x 1.75 cm were taken from digital images of each culture

204 plate: one section was taken from close to the bacterial growth (query bacteria) and two from

205 sections were only *Bd* was present (control section of the plate). To estimate the antifungal action

206 of bacteria, a graphical approach was developed using programming routines for R software (R

207 Core Team, 2016). The three images were read into R using the function *raster* of 'raster'

208 package (Hijmans, 2015) and then a frequency table was created based on the color present in

209 each pixel using the function *freq* of the same package. We generated a continuous color scale

210 ranging from 0 (white – representing only presence of *Bd*) to 255 (black – representing complete

211 absence of *Bd*). To ensure these extreme values were present in the picture, two circles of equal

212 dimensions (one of each color) were manually added to each image.

213

214 Because *Bd* does not grow uniformly over the plate's surface, two control assessments were

215 employed: a) We obtained kernel density estimates for each control picture and the average of

216 the modal color value from each picture was used in all posterior analyses. For this step, we used

217 the function *density* from the 'stats' package in R (R Core Team, 2016) with its default

218 arguments, and, b) the frequency tables of each control picture were averaged by color, and then

219 the color value corresponding to the mode of the kernel density estimates was used as control.

220 These two control values were compared to the modal color value of the picture showing the

221 bacterial effect on *Bd* growth. We assumed that lower experimental values than the control imply

222 *Bd* inhibition (darker background in picture), and no differences between control and

223 experimental values imply no antifungal effect (S1).

224

225   *Identification of bacterial isolates*

226   DNA from pure cultures, including inhibitory and non-inhibitory strains were extracted as

227   follows: one colony of each morphotype was re-suspended in 10 mL of pure water HPLC grade

228   in a 0.2 mL PCR tube and boiled for 7 min at 95 ºC. PCR amplifications were performed using

229   universal primers 27F and 1492R (Lane, 1991) and the following parameters: an initial

230   denaturation of 3 min at 95 ºC, followed by 35 cycles of 45 s at 95 ºC, 45 s at 52 ºC and 90 s at

231   72 ºC, and a final extension of 7 min at 72 ºC. Each reaction consisted of 0.5 µL of each primer

232   (1 µM), 3.0 µL of doubly distilled DNA-free water, 6 µL of GoTaq® Green Master Mix (1X;

233   Promega) and 2 µL of the DNA extract. PCR results were checked by electrophoresis in 1%

234   agarose gels. DNA sequences were cleaned and assembled using Geneious (Drummond et al.,

235   2011). 16S rRNA sequences were then identified using BLASTn (Altschul et al., 1990) against

236   the complete GenBank nucleotide database (http://www.ncbi.nlm.hih.gov) and the Greengenes

237   database (http://greengenes.lbl.gov) using default parameters in both cases.

238

239   *Statistical analyses*

240   In order to determine the variable (species or condition) that best explained the composition of

241   the skin-associated bacterial community we used a Canonical Correspondence Analysis - CCA

242   using the function *cca* in the 'vegan' package in R (Oksanen, 2015). In addition, to compare

243   bacterial diversity between wild and captive animals, we counted the number of bacterial species

244   in each category (species and condition) and then we performed a chi-square test using the 'stats'

245   package in R (R Core Team, 2016). In order to determine the effect of captivity on bacterial

246   assemblages, we used a chi-square test to check if there were differences in the proportion of

247   bacterial species with antibacterial properties between conditions (wild vs. captive). Lastly, to

248   test for differences in the capacity of bacterial species to inhibit *Bd* depending on their origin

249   (wild vs. captive), we performed an ANOVA using the data obtained from the image analysis,

250   after testing for normality and homogeneity of variances using Shapiro-Wilk and Bartlett tests,

251   respectively.

252

253

254   **Results**

255

256   *Isolated bacteria from wild and captive individuals*

257   Based on morphological characteristics, a total of 153 bacterial morphotypes were isolated from

258   captive individuals: 70 from *A. elegans*, 62 from *A.* aff. *limosus*, and 21 from *A. spurrelli*. We

259   recovered 40 morphotypes from wild *A. elegans*. Fifteen (21%) bacterial morphotypes from

260   captive and 11 (27%) from wild individuals of *A. elegans* inhibited *Bd* growth in the antagonism

261   assays. For captive *A.* aff. *limosus* and *A. spurrelli*, 29 of 62 and six of 21 morphotypes inhibited

262   *Bd* growth, respectively (Table 2). Since identification of bacteria based only on morphology is

263   not completely reliable, we sequenced the 16S rRNA gene for all the isolated morphotypes.

264   From 193 culturable morphotypes, we were able to identified bacteria representing 74 OTUs

265   belonging to 37 genera. Through antagonism assays we found 28 OTUs (38%) in 13 genera

266   showed some degree of anti-*Bd* activity. To define bacterial species we used a cut of 97%

267   similarity. Sequences are available in GenBank with the following accession numbers:

268   KY910042-KY9110115 and KY938077-KY938172.

269

270   *Effect of captivity on the composition of skin bacterial community*

271   Although captive and wild individuals from the same host species harbor cutaneous bacteria

272   belonging to the same genera (Fig. 2), we found little overlap in bacterial species diversity

273   between conditions (CCA: $\chi^2_{df=1}$ = 0.408, F = 1.23, P = 0.02) (Table 3, Fig. 3). We did not find

274   evidence for reduction in the number of bacterial species between wild and captive animals

275   ($\chi^2_{df=2}$ = 3.6551, P = 0.1608, Fig. 4). Antagonism experiments revealed that animals in the wild

276   and individuals kept in *ex situ* facilities harbor in their skin bacteria with antifungal capacities.

277   Differences in the proportion of bacteria with antifungal abilities between captive (22/64) and

278   wild individuals (18/53) were not significant (Fisher exact test, P = 1, Odds ratio=1.01, Fig. 4).

279

280   *Anti-*Bd *activity in shared bacterial strains*

281   To determine if anti-*Bd* activity changes depending on the origin of the bacteria, we compared

282   the ability to inhibit *Bd* only for those bacterial species that were found in both captive and wild

283   animals. We detected five bacterial species that inhibited *Bd* growth in both conditions including:

284   *Acinetobacter* sp., *Chryseobacterium* sp., *C. meningosepticum*, *Pseudomonas putida*, and

285   *Pseudomonas* sp. The inhibition was measured as a reduction of density of *Bd* colonies (based on

286   the presence of darker pixels in the experimental area). Differences in the extent of inhibition

287   between bacteria isolated from wild and captive animals were not detected (F=0.503, P=0.73;

288   Fig. 5). Bacteria inhibit *Bd* growth irrespective of the *Bd* strain used (JEL or EV001, Fisher exact

289   test, P=0.051, Odds ratio=0.385).

290

291

292   **Discussion**

293

294   The genus *Atelopus* is one of the most threatened groups of amphibians worldwide. Although

295   more than 70% of the species in the genus are listed as critically endangered according to the

296   IUCN (2016), some species have persisted in the wild (Flechas, Vredenburg & Amézquita, 2015).

297   In other cases, such as *A. elegans* from Ecuador, this species has been recently rediscovered but

298   only in one of the historical localities. Given the high susceptibility of the genus to

299   chytridiomycosis, and the lack of a cure that could allow them to survive in nature, conservation

300   actions have been focused on keeping assurance colonies. In this study, we describe culturable

301   bacterial assemblages for individuals of three *Atelopus* species that remain in the wild, in

302   apparently healthy conditions, and individuals (from the same species and collection localities)

303   brought into captivity. This study sought to determine the effect of captive conditions on the anti-

304   *Bd* bacterial community in one of the most threatened genera worldwide (La Marca et al., 2005;

305   Mendelson et al., 2006). Our main goal was not only to describe the culturable portion of the

306   bacterial community for each condition, but the effect of captivity on the proportion of beneficial

307   bacteria and their performance as *Bd* inhibitors. Although our results show that captive

308   individuals harbor bacteria with anti-*Bd* properties, further long-term research is key to evaluate

309   the interactions between the skin microbial communities and their amphibian hosts since those

310   can differ from one population to the next even if the bacterial community composition is similar

311   (Rebollar et al., 2016).

312

313   Several studies have reported changes in the microbial associated communities when animals are

314   moved from the wild to captive environments (Isaacs et al., 2009; Dhanasiri et al., 2011;

315   Wienemann et al., 2011; Nelson et al., 2013). In amphibians, research assessing the effect of

316   captivity on bacterial communities is increasing (Antwis et al., 2014; Becker et al., 2014;

317   Michaels, Antwis & Preziosi, 2014a; Kueneman et al., 2016). Through culture-independent and

318   dependent approaches, these studies have demonstrated changes in the skin bacterial community

319   composition due to conditions imposed by captivity. Our results suggest that *ex situ* conditions,

320   at least for these three *Atelopus* species, did not reduce the diversity of bacteria or the proportion

321   of anti-*Bd* bacteria, as determined by culture-based assays. Moreover, we found a higher number

322   of culturable bacterial species in captive animals compared to wild individuals. Our findings are

323   consistent with those obtained for other vertebrates including birds and mammals (Xenoulis et al.,

324   2010; Nelson et al., 2013;) where gut microbial diversity was higher in captive animals, and are

325   also consistent with the findings for the Panamanian golden frogs (*A. zeteki*) where OTU

326   richness and phylogenetic diversity was higher in captive frogs (Becker et al., 2014). We found

327   that species of bacteria belonging to the genera *Pseudomonas, Chryseobacterium* and

328   *Acinetobacter* isolated from wild and captive animals inhibited *Bd* in the challenge assays. In a

329   recent study, bacterial isolates from these three groups were also reported as *Bd* inhibitors and

330   they appeared to be widely distributed. The proportion of bacteria that showed anti-*Bd* activity

331   for those genera was 73% for *Pseudomonas,* 55% for *Chryseobacterium* and 37% for

332   *Acinetobacter* (Woodhams et al., 2015).

333

334   Variations in bacterial communities due to captive conditions have been demonstrated in red-

335   backed salamanders (*Plethodon cinereus*), where environmental microbes seemed to affect host-

336   microbial diversity and appeared to be important as regulators of the host core community

337   (Loudon et al., 2014). In terms of their associated microbiota, captive environments appear to be

338   less diverse and less heterogeneous than wild habitats, which might imply that host associated

339   bacterial communities for animals in *ex situ* facilities should be less diverse (Sabino-Pinto et al.,

340   2016). The enclosures where frogs are maintained provide a modified environment that can also

341   vary depending on the zoo or facility. These conditions could facilitate the transmission of new

342   bacteria capable of colonizing the frog skin, as suggested for other animals in captivity (Nelson

343   et al., 2013). We found that culturable bacterial communities from captive animals at the Cali

344   Zoo (*A*. aff. *limosus* and *A. spurrelli*) were not the same but more similar to each other than

345   compared with *A. elegans*. The slight difference between captive *A*. aff. *limosus* and *A. spurrelli*

346   (Fig. 2), suggests that a "host" component is also playing a role shaping skin bacterial

347   communities, as suggested previously for Japanese amphibians (Sabino-Pinto et al., 2016).

348   Although the cages used in this study simulated natural conditions, we propose that the observed

349   differences between frogs from both *ex situ* facilities might be related to the environmental

350   conditions inside the enclosures (i.e., substrate and water sources). Although, there is no solid

351   evidence on which mechanisms are the frogs using to acquire bacteria from the environment, and

352   which are the key factors modeling bacterial assemblages, we suggest that either the environment

353   and host characteristics are modeling bacterial communities in these species of harlequin toads.

354

355   A cure or mechanism that allows threatened amphibian species to survive chytridiomycosis in

356   their natural habitats is still needed. Although many different disease mitigation strategies have

357   been proposed (Woodhams et al., 2011; Bosch et al., 2015), keeping viable assurance colonies in

358   captivity seems to be the best option for *Bd*-susceptible species yet (Gratwicke et al., 2015). The

359   main goal of *ex situ* programs is to eventually reintroduce animals to their natural habitats.

360   Management in captivity can lead to alterations of the species' microbiota that may reduce

361   survival chances after reintroduction (Redford et al., 2012). In this study, we isolated a similar

362   number of bacterial species with anti-*Bd* activity from captive and wild individuals of three

363   *Atelopus* species, suggesting that they might count on their skin bacteria as a potential defense

364   barrier against *Bd*. Redford et al. (2012) proposed that more studies are needed to determine

365   changes and alterations in the animals' microbiome in order to increase the success of

366   reintroduction efforts. Here, we presented evidence that although bacterial community structure

367   changed in captivity, the predicted anti-*Bd* function remained intact. In addition, it seems that

368   animals are acquiring new microorganisms that are also providing protection against the fungal

369   pathogen. Our findings suggest that selection acted on bacterial community function (e.g.,

370   antifungal activity and protection) as opposed to selection for specific community structures. We

371   hope this study might be considered when reintroduction plans are proposed as the next step for

372   many species that are now part of *ex situ* programs.

373

374

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376

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570

Figure 1

Sampling sites

Map showing the localities where bacterial samples were obtained *Atelopus* aff. *limosus* (green dot), *Atelopus spurrelli* (blue triangle) *Atelopus elegans* (red square). Individuals brought to captivity were collected in the same localities where samples from wild individuals were obtained. The map was created using the profiles provided by GADM.

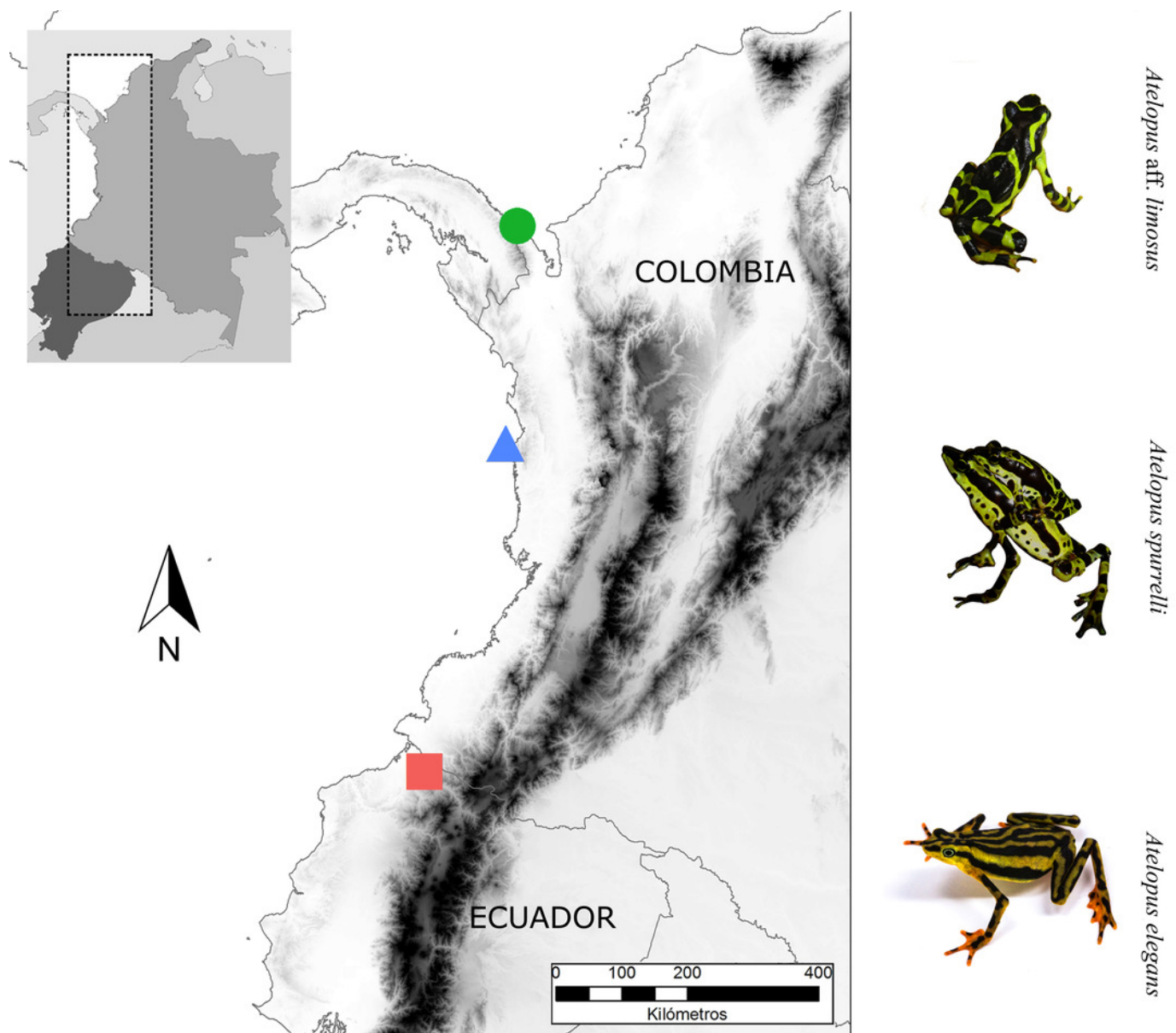



Figure 2

Differences in the relative abundance of culturable bacterial genera isolated from the skin of captive and wild individuals in three species of *Atelopus.*

Bars are grouped by species and condition. Numbers inside the bars denote the number of toads sampled per category. C = Captive, W = Wild.

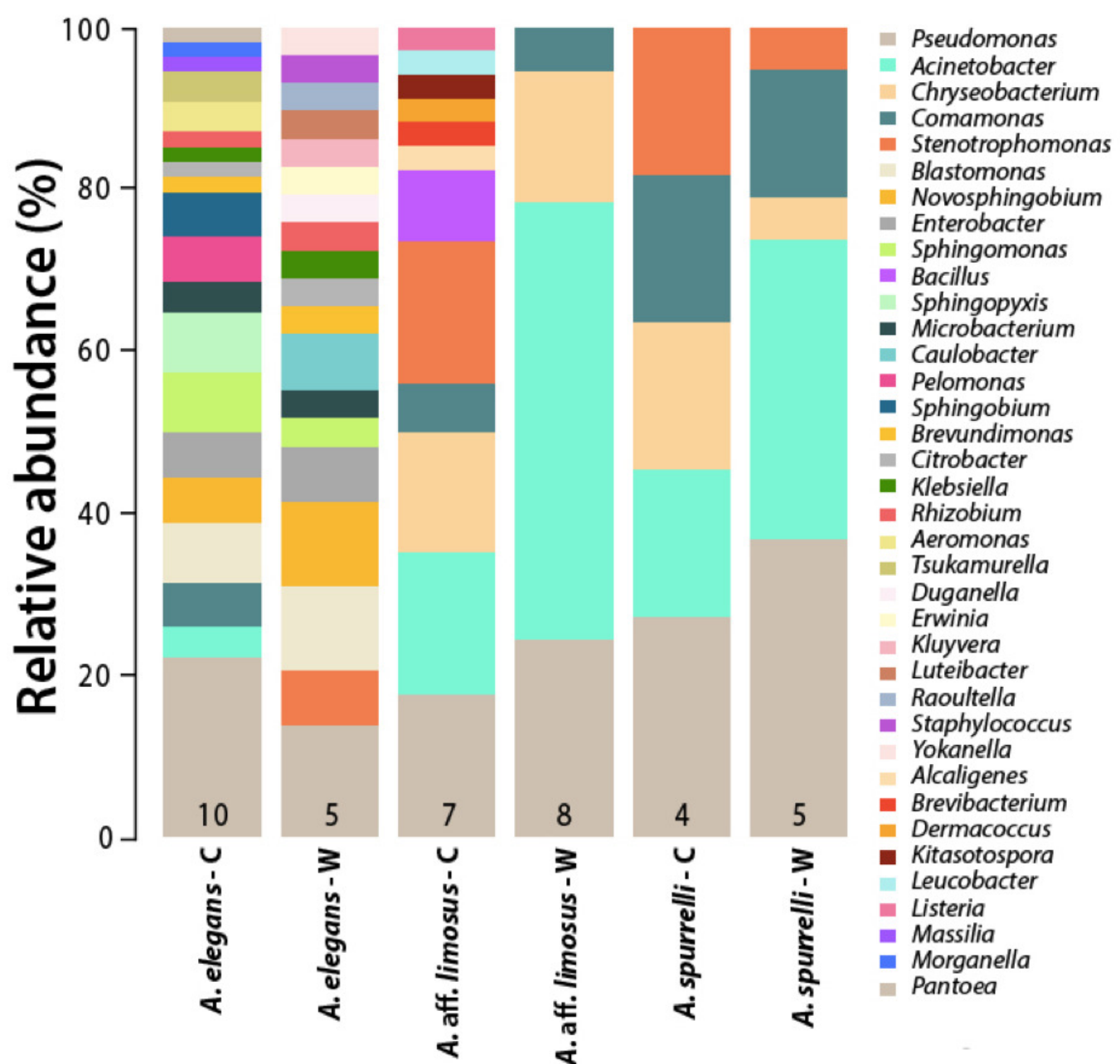



Figure 3

Culturable bacterial communities between captive and wild individuals of *Atelopus* exhibit little overlap.

Non-metric Multidimensional Scaling (NMDS) ordination of Bray-Curtis distances between microbial communities. Each symbol represents one sampled individual. Ellipses represent 95% confidence intervals (Dark grey = wild, light grey = captive).

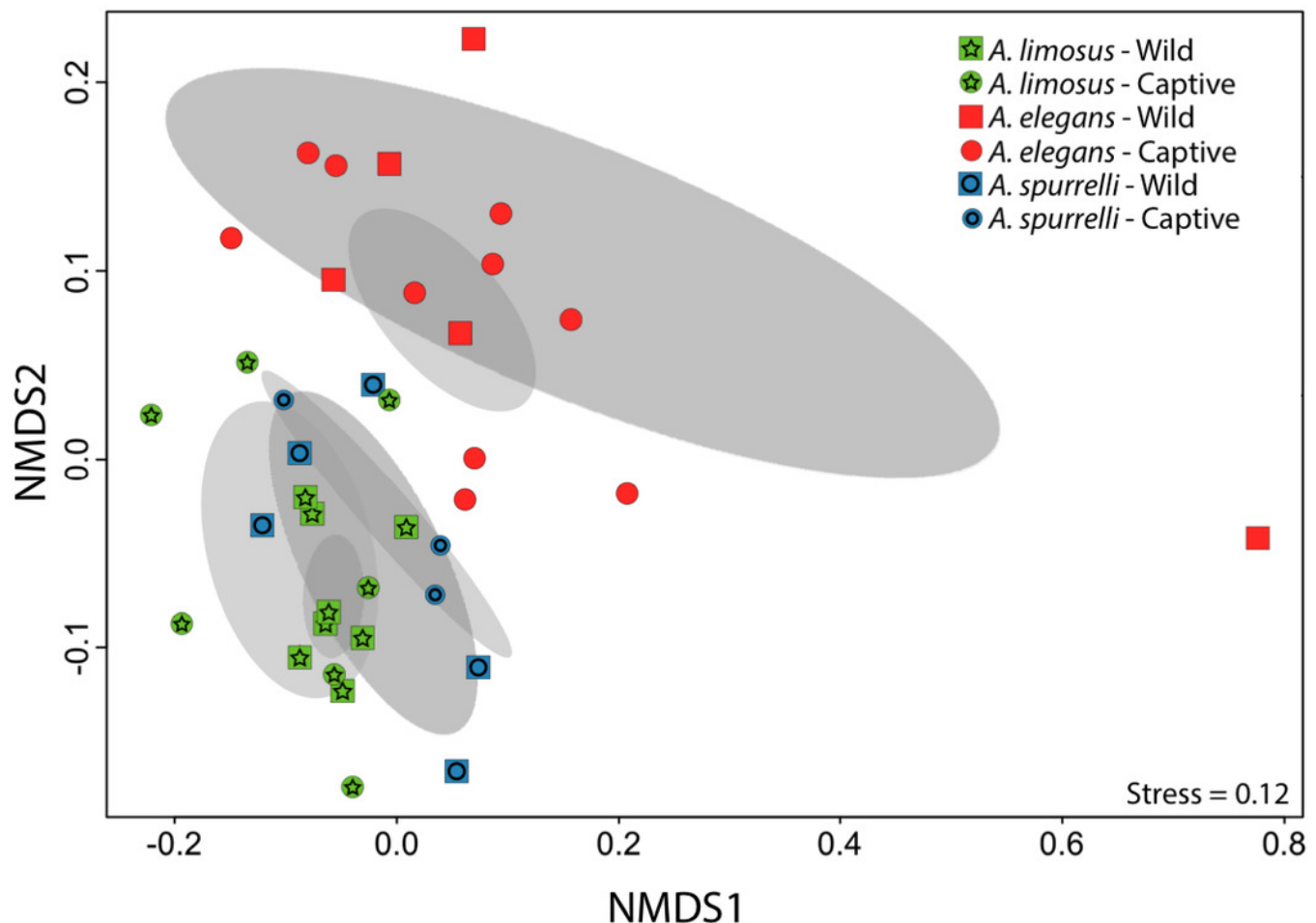



Figure 4

Atelopus in the wild and in captivity harbor in their skin culturable bacteria with antifungal abilities.

Darker bars show the total number of isolated bacteria in each condition (wild vs. captive). Lighter bars represent the number of bacterial species with anti-Bd activity in each condition (wild vs. captive).

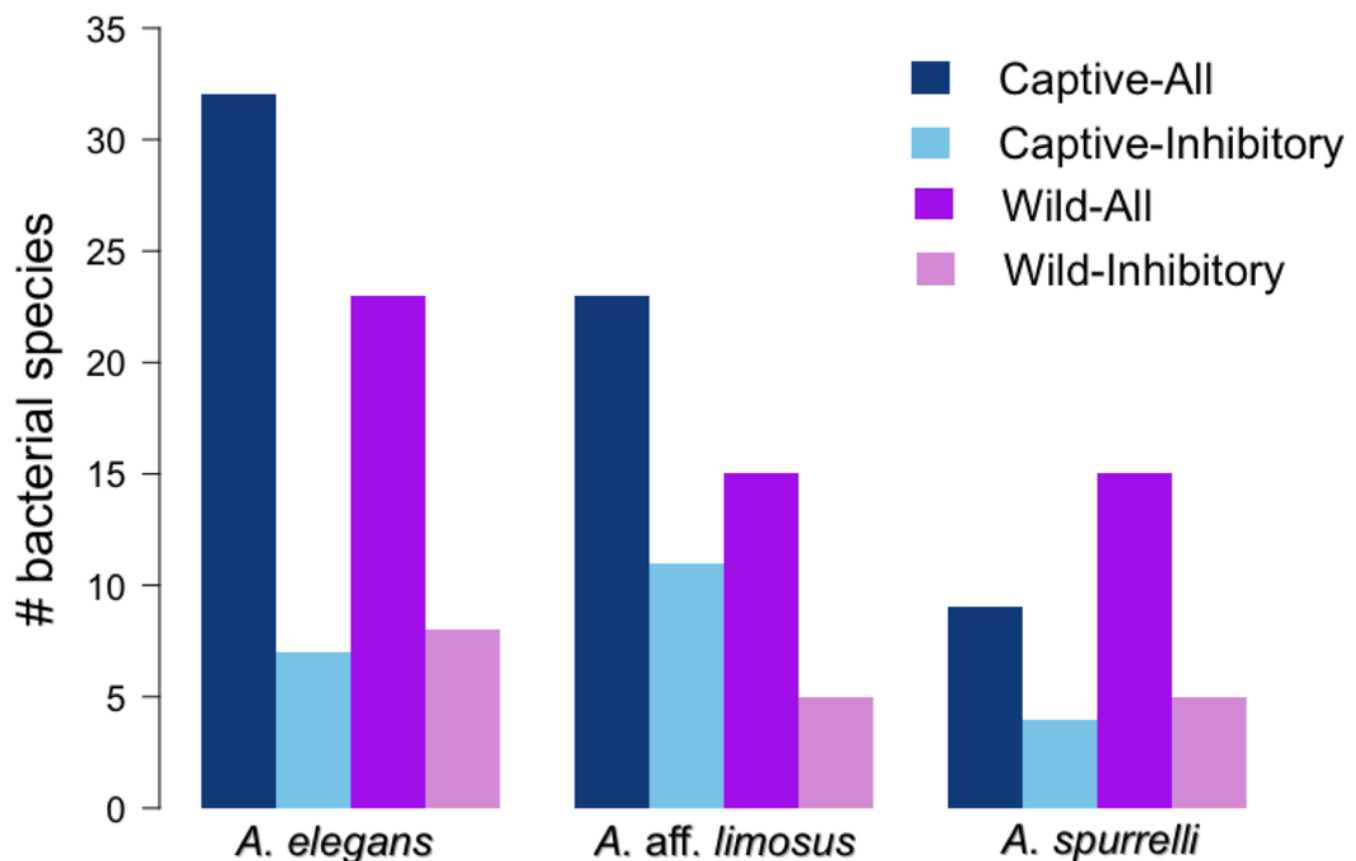
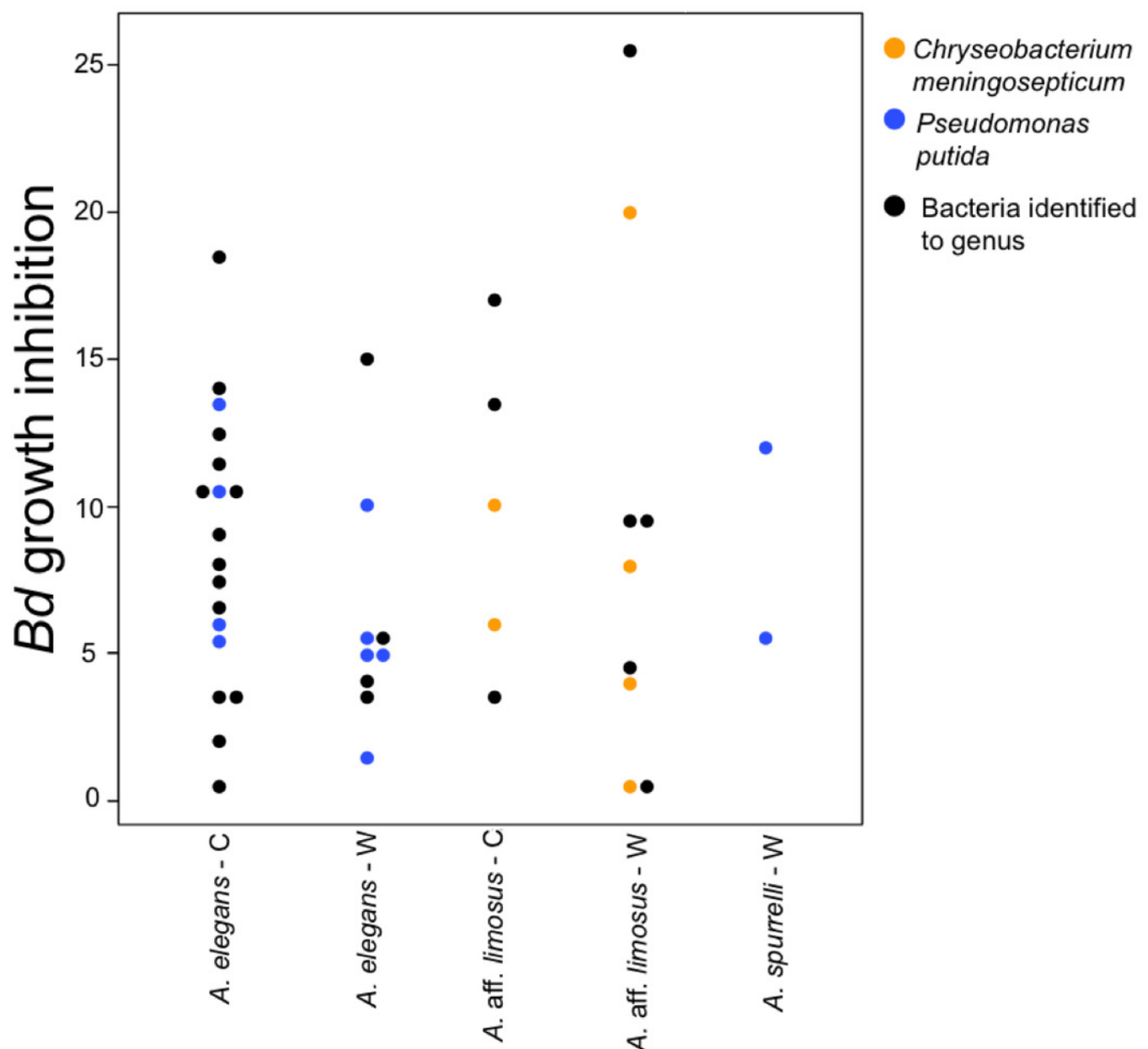



Figure 5

Bacteria isolated from wild and captive animals did not differ in the extent of *Bd* inhibition.

Colored dots were only used for those bacteria that were identified to species. Black dots correspond to the bacteria that were identified to genus.

Number of sampled individuals of each *Atelopus* species in wild and captive conditions

The table includes the localities in Colombia and Ecuador where *Atelopus* individuals were sampling, the year when individuals were collected and entered into an ex situ facility, as well as the year when bacterial samples were taken for the analysis of the culturable portion of the skin microbiota.

Species	Site of collection	Stage	captive ind.	Year of collection	Date of sampling captive individuals	wild ind.	Date of sampling wild individuals
Atelopus elegans	Esmeraldas, Ecuador	Juvenile	2	1 ind - 2009 1 ind - 2010	2012	-	-
		Adult	8	2 ind - 2009 3 ind - 2010 1 ind - 2011 2 ind - 2012	2012	5	2012
Atelopus aff. limosus	Capurganá, Colombia	Juvenile	2	Born in captivity in 2011	2012	-	-
		Adult	5	2 ind - 2008 3 ind -2009	2012	8	2009
Atelopus spurrelli	Arusí, Colombia	Adult	4	2011	2012	5	2009

Summary of the number of bacterial isolates recovered from three *Atelopus* species in captive and wild conditions

The number in parenthesis represents the percentage of isolates with anti-*Bd* properties. In the last column we detailed the number of bacterial species that exhibit some degree of anti-*Bd* activity.

Species	Condition	# of isolates	# Anti-*Bd* isolates	# Bacterial species with anti-*Bd* activity
Atelopus elegans	Captive	70	15 (21%)	7 from 3 genera
	Wild	40	11 (27%)	9 from 7 genera
Atelopus aff. *limosus*	Captive	62	29 (47%)	11 from 6 genera
	Wild	77	20 (26 %)	7 from 3 genera
Atelopus spurrelli	Captive	21	6 (28%)	4 from 4 genera
	Wild	21	6 (28%)	5 from 2 genera

Bacterial species isolated from the skin of *Atelopus elegans*, *Atelopus* aff. *limosus* and *Atelopus spurrelli*

C=Captive, W=Wild. The number in parenthesis represents the number of individuals sampled in each condition. Numbers in each cell represent the number of isolates for each bacterial species. Species that showed anti-*Bd* activity are marked with an asterisk (*). Bacteria highlighted in grey correspond to those present in captive and wild animals.

Family	Bacterial Isolates	*A. elegans*		*A.* aff. *limosus*		*A. spurrelli*	
		C (10)	W (5)	C (7)	W (8)	C (4)	W (5)
Moraxellaceae	*Acinetobacter baumannii*	-	-	1	3	-	2
Moraxellaceae	*Acinetobacter bereziniae*	-	-	1	-	-	-
Moraxellaceae	*Acinetobacter calcoaceticus*	-	-	1	2	-	1
Moraxellaceae	*Acinetobacter genomosp.*	-	-	-	1	-	-
Moraxellaceae	*Acinetobacter haemolyticus*	-	-	-	-	-	1*
Moraxellaceae	*Acinetobacter gyllenbergii*	-	-	-	5*	-	1*
Moraxellaceae	*Acinetobacter junii*	-	-	-	1	-	-
Moraxellaceae	*Acinetobacter* sp.	2	-	3*	7*	2	2*
Moraxellaceae	*Acinetobacter venetianus*	-	-	-	1	-	-
Aeromonadaceae	*Aeromonas hydrophilia*	1	-	-	-	-	-
Aeromonadaceae	*Aeromonas* sp.	1	-	-	-	-	-
Alcaligenaceae	*Alcaligenes faecalis*	-	-	1*	-	-	-
Bacillaceae	*Bacillus* sp.	-	-	1	-	-	-
Bacillaceae	*Bacillus cereus*	-	-	2*	-	-	-
Sphingomonadaceae	*Blastomonas natatoria*	4	3*	-	-	-	-
Brevibacteriaceae	*Brevibacterium aureum*	-	-	1	-	-	-
Caulobacteraceae	*Brevundimonas aurantiaca*	1	1	-	-	-	-
Caulobacteraceae	*Caulobacter crescentus*	-	1	-	-	-	-
Caulobacteraceae	*Caulobacter vibroides*	-	1	-	-	-	-
Flavobacteriaceae	*Chryseobacterium indologenes*	-	-	2*	-	-	-
Flavobacteriaceae	*Chryseobacterium meningosepticum*	-	-	1*	1*	2*	1
Flavobacteriaceae	*Chryseobacterium* sp.	-	-	2*	5*	-	-
Enterobacteriaceae	*Citrobacter freundii*	-	1*	-	-	-	-
Enterobacteriaceae	*Citrobacter* sp.	1*	-	-	-	-	-
Comamonadaceae	*Comamonas* sp.	3	-	-	1	1	1
Comamonadaceae	*Comamonas testosteroni*	-	-	2	1	1*	2
Oxalobacteraceae	*Duganella* sp.	-	1	-	-	-	-
Dermacoccaceae	*Dermacoccus* sp.	-	-	1	-	-	-
Enterobacteriaceae	*Enterobacter asburiae*	1*	-	-	-	-	-
Enterobacteriaceae	*Enterobacter* sp.	2	2	-	-	-	-
Enterobacteriaceae	*Erwinia amylovora*	-	1	-	-	-	-
Streptomycetaceae	*Kitasatospora phosalacinea*	-	-	1	-	-	-
Enterobacteriaceae	*Klebsiella oxytoca*	1	-	-	-	-	-
Enterobacteriaceae	*Klebsiella* sp.	-	1	-	-	-	-
Enterobacteriaceae	*Kluyvera ascorbata*	-	1*	-	-	-	-
Microbacteriaceae	*Leucobacter* sp.	-	-	1	-	-	-
Listeriaceae	*Listeria* sp.	-	-	1	-	-	-
Xanthomonadaceae	*Luteibacter rhizovicinus*	-	1*	-	-	-	-
Oxalobacteraceae	*Massilia* sp.	1	-	-	-	-	-
Microbacteriaceae	*Microbacterium foliorum*	1	-	-	-	-	-
Microbacteriaceae	*Microbacterium* sp.	1	-	-	-	-	-
Microbacteriaceae	*Microbacterium testaceum*	-	1	-	-	-	-
Enterobacteriaceae	*Morganella morganii*	1	-	-	-	-	-
Sphingomonadaceae	*Novosphingobium aromaticivorans*	1	-	-	-	-	-
Sphingomonadaceae	*Novosphingobium subterraneum*	2	3*	-	-	-	-
Enterobacteriaceae	*Pantoea agglomerans*	1	-	-	-	-	-
Comamonadaceae	*Pelomonas puraquae*	3	-	-	-	-	-
Pseudomonadaceae	*Pseudomonas aeruginosa*	1*	-	-	-	-	-

Pseudomonadaceae	*Pseudomonas fulva*	1	**1***	-	-	-	-
Pseudomonadaceae	*Pseudomonas geniculata*	-	-	**1***	-	-	-
Pseudomonadaceae	*Pseudomonas montielli*	-	-	-	-	**1***	-
Pseudomonadaceae	*Pseudomonas mosselii*	1*	-	1*	-	-	-
Pseudomonadaceae	*Pseudomonas plecoglossicida*	-	-	1	-	-	**1***
Pseudomonadaceae	*Pseudomonas putida*	**2***	**1***	**2***	1	1	**2***
Pseudomonadaceae	*Pseudomonas saccharophilia*	1	-	-	-	-	-
Pseudomonadaceae	*Pseudomonas* sp.	**4***	**2***	2	1	1	1
Pseudomonadaceae	*Pseudomonas nitroreducens*	-	-	-	-	-	1
Pseudomonadaceae	*Pseudomonas straminea*	-	-	-	-	-	1
Pseudomonadaceae	*Pseudomonas tolaasii*	-	-	-	1	-	1
Pseudomonadaceae	*Pseudomonas veronii*	-	-	-	**6***	-	-
Pseudomonadaceae	*Pseudomonas vranovensis*	**2***	-	-	-	-	-
Enterobacteriaceae	*Raoultella ornithinolytica*	-	1	-	-	-	-
Rhizobiaceae	*Rhizobium* sp.	1	1	-	-	-	-
Sphingomonadaceae	*Sphingobium yanoikuyae*	2	-	-	-	-	-
Sphingomonadaceae	*Shipngomonas paucimobilis*	1	-	-	-	-	-
Sphingomonadaceae	*Sphingomonas* sp.	4	1	-	-	-	-
Sphingomonadaceae	*Sphingopyxis* sp.	4	-	-	-	-	-
Staphylococcaceae	*Staphylococcus capitis*	-	1	-	-	-	-
Xanthomonadaceae	*Stenotrophomonas maltophilia*	-	1	**4***	-	1	1
Xanthomonadaceae	*Stenotrophomonas* sp.	-	1	**2***	-	**1***	-
Nocardiaceae	*Tsukamurella* sp.	1	-	-	-	-	-
Nocardiaceae	*Tsukamurella tyrosinosolvens*	1	-	-	-	-	-
Enterobacteriaceae	*Yokenella regensburgei*	-	1	-	-	-	-