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Genotyping and differential bacterial inhibition of *Batrachochytrium dendrobatidis* in threatened amphibians in Costa Rica

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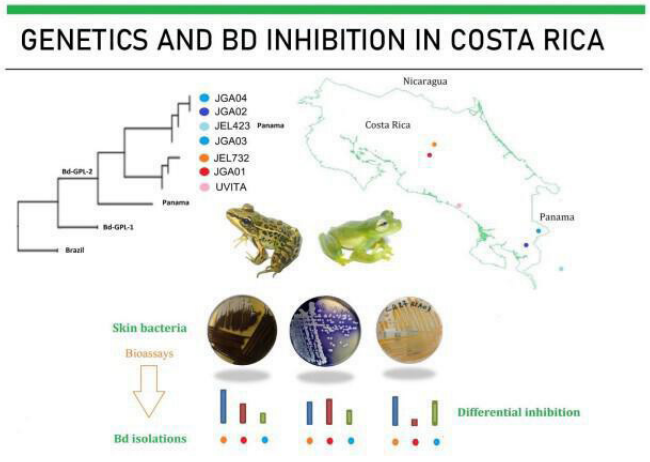
Abstract

Amphibians have declined around the world in recent years, in parallel with the emergence of an epidermal disease called chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). This disease has been associated with mass mortality in amphibians worldwide, including in Costa Rica, and *Bd* is considered an important contributor to the disappearance of this group of vertebrates. While many species are susceptible to the disease, others show tolerance and manage to survive infection with the pathogen. We evaluated the pathogen *Bd* circulating in Costa Rica and the capacity of amphibian skin bacteria to inhibit the growth of the pathogen *in vitro*. We isolated and characterized – genetically and morphologically – several *Bd* isolates from areas with declining populations of amphibians. We determined that the circulating chytrid fungus in Costa Rica belongs to the virulent strain *Bd*-GPL-2, which has been related to massive amphibian deaths worldwide; however, the isolates obtained showed genetic and morphological variation. Furthermore, we isolated epidermal bacteria from 12 amphibian species of surviving populations, some in danger of extinction, and evaluated their inhibitory activity against the collection of chytrid isolates. Through bioassays we confirmed the presence of chytrid-inhibitory bacterial genera in Costa Rican amphibians. However, we observed that the inhibition varied between different isolates of the same bacterial genus, and each bacterial isolation inhibited fungal isolation differently. In total, 14 bacterial isolates belonging to the genera *Stenotrophomonas*, *Streptomyces*, *Enterobacter*, *Pseudomonas* and *Klebsiella* showed inhibitory activity against all *Bd* isolates. Given the observed variation both in the pathogen and in the bacterial inhibition capacity, it is highly relevant to include local isolates and to consider the origin of the microorganisms when performing *in vivo* infection tests aimed at developing and implementing mitigation strategies for chytridiomycosis.



Graphical abstract

All Bd *Batrachochytrium dendrobatidis* isolates in Costa Rica belong to the Bd-GPL-2 lineage. Amphibian skin bacteria can inhibit the growth of *Bd* to different degrees, depending on the chytrid isolate.

INTRODUCTION

Amphibians have suffered catastrophic losses of biodiversity around the world: more than 32% of the nearly 8000 known species are threatened [1, 2]. Costa Rica's herpetofauna has been one of the best studied on the planet [3], and also one that has experienced considerable loss of amphibian species [4]. Currently, 64 species of Costa Rican amphibians are in a degree of threat according to the IUCN [1]. Several factors contribute to amphibians being globally threatened, including habitat destruction, invasive species, climate change, chemical pollutants and infectious diseases such as chytridiomycosis [5–7]. Chytridiomycosis is a panzootic disease associated with the fungus *Batrachochytrium dendrobatidis* (*Bd*) that has been detected in sites with important amphibian declines [8, 9]. It is considered one of the worst infectious diseases among vertebrates in terms of the number of species affected [2, 8] (see Lambert *et al.* [10] for a discussion).

Although *Bd* was discovered in 1998 [11], molecular studies of museum specimens found evidence of its presence in amphibian samples more than 100 years old [12, 13], demonstrating its association with amphibians long before population declines. In Costa Rica, *Bd* has a wide geographical and environmental distribution [14–16], and based on quantitative PCR tests the earliest specimen that tested positive for *Bd* dates from 1964 [17]. However, it has been determined that *Bd* has several lineages distributed around the world, including one hypervirulent strain (*Bd*-GPL) [18–22, 23]. In addition to genetic differences in *Bd* lineages, differences in morphology including growth rate, size of sporangia, spore size, zoospore production and protein production have been described [24, 25]. Furthermore, positive correlations have been reported between the size of the sporangia and spores and the prevalence and intensity of infection [19, 22, 25].

Chytridiomycosis can be controlled by treating infected amphibians with heat or with the antifungal itraconazole [26]. Itraconazole has been used in captivity with mixed results [27], as it involves submerging the amphibians repeatedly in the antifungal solution, which produces a stress response in the individual that can cause harmful side effects [26]. Mitigating chytridiomycosis in a natural environment is very difficult. Only a single case of effective mitigation has been described so far [28], but it would be extremely difficult to replicate in most natural ecosystems because this procedure requires the removal of all individuals from the environment. Several lines of evidence suggest that *Bd* is an opportunistic pathogen, because it can be present in healthy amphibians [29], while in susceptible individuals that fail to eliminate the infection the clinical symptoms of the disease appear [7, 11].

An amphibian species is susceptible to chytridiomycosis if populations have high mortality and individuals fail to overcome the infection [7]. An example of a highly susceptible species is the harlequin toad of the genus *Atelopus* [30]. On the other hand, a species is considered tolerant if it has the capacity to limit the consequences of the infection; in this case, although the pathogen is present, the animals do not die from chytridiomycosis [7]. Although the decline of amphibians observed in the 1980s and 1990s led to their disappearance, several species of amphibians have been rediscovered since the year 2000 [31–39]. It remains unclear why certain populations or species of amphibians survive whereas others are extirpated with the appearance of *Bd*. Although there is currently no definitive answer to this, several hypotheses have been proposed: differential virulence of the *Bd* lineages [19, 21, 40], innate immunity [41, 42], adaptive immunity [43–45], behaviours that favour the clearing of the pathogen [46–48], climatic refuges [37] and *Bd* inhibition by skin microbes that help innate and acquired immunity [49].

Amphibians have bacteria on their skin capable of inhibiting *Bd* in the laboratory [50, 51]. Various bacterial genera are capable of inhibiting the growth of *Bd* [50, 52, 53, 54, 55]. The inhibitory properties of these bacterial genera are related to the production of certain secondary metabolites such as indole-3-carboxaldehyde, violacein and prodigiosin [52]. The use of these bacteria as probiotics is a promising strategy, but to date the effectiveness of this treatment is not well known [56]. Various studies have been carried out applying one or more bacteria both in the wild and in captivity with contradictory results; in some cases it helps survival of the species [57] but not in others [58] and applying the same bacteria to different frog species has not always had the same effect [59]. At present, it is still unclear which bacteria or consortia could be more effective [60] and what the effectiveness is when used on live amphibians [58]. For that reason, there is a need to isolate bacteria that meet certain requirements to be employed as probiotics [61–63]. The candidate probiotics should be present in diverse species, have a wide geographical distribution, protect several hosts at temperatures consistent with the host habitat, be harmless to the host, other organisms or environment, and able to persist in the presence of *Bd* and the immune defence of the host skin [62–64].

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Abbreviations: *Bd*, *Batrachochytrium dendrobatidis*.
The GenBank accession numbers for the 16S rRNA gene sequences are: MN829883–MN829941.
Three supplementary tables and two supplemenrtary materials are available with the online version of this article.

Table 1. Costa Rican localities visited for the isolation of the pathogen (a) and for obtaining cutaneous bacteria (b)

Location	Latitude north	Longitude west	Altitude (m)
Santo Domingo, Bougainvillea Hotel, Heredia (a, b)	10.0°	84.1°	1170
Punta Banco, Golfito, Puntarenas (a, b)	8.4°	83.1°	20
Río Murciélago, Peninsula of Santa Elena, Guanacaste (b)	10.9°	85.7°	100
Santa Elena Reserve, Monteverde, Puntarenas (b)	10.3°	84.8°	1200
La Selva Biological Station, Sarapiquí, Heredia (a, b)	10.4°	84.0°	100
Cerro Chompipe, Braulio Carrillo National Park, Heredia (b)	10.1°	84.1°	2100
Veragua Rain Forest Adventure, Talamanca, Limón (b)	9.9°	83.2°	300
Las Cruces Biological Station, San Vito, Puntarenas (a, b)	8.8°	83.0°	1200
El Tirol, Whitworth University Campus, Heredia (a)	10.1°	84.1°	1800
Guayacán, Costa Rican Amphibian Research Center, Siquirres, Limón (a)	10.1°	83.5°	533
Las Tablas Protected Zone, San Vito de Coto Brus, Puntarenas (a)	8.9°	82.8°	1350
Uvita de Osa, Dominical, Puntarenas (a)	9.1°	83.7°	400
Rancho Chutas, Children's Eternal Rainforest, Alajuela (b)	10.4°	84.7°	1763

The present work aims to bridge information gaps related to chytridiomycosis in Costa Rica and Central America. The presence and prevalence of the pathogenic fungus have been well documented within Costa Rica [14–17, 65] but which genotypes are circulating has not been determined. Reports of mass amphibian deaths in the country [66] suggest that the circulating pathogen belongs to the hypervirulent *Bd*-GPL strain. However, survival of some populations of highly *Bd*-susceptible amphibian species may suggest that endemic or non-virulent lineages of *Bd* may be present as well. Additionally, the tolerance of some amphibian species could be provided by cutaneous bacteria that inhibit the growth of the pathogen [61]. It is unknown whether the amphibian populations surviving declines in Costa Rica have bacteria capable of inhibiting different local lineages of the pathogenic fungus. The main objective of this work is to characterize the genetics of the *Bd* fungus and identify *Bd*-inhibitory bacteria of Costa Rican amphibian species, in order to obtain a better understanding of disease dynamics in the country.

METHODS

Study site and field sampling

Field sampling took place during 15 field visits of 1–5 days each between 2012 and 2016 [65]. For isolation of the *Bd* pathogen and cutaneous amphibian bacteria, 13 localities were visited in Costa Rica, as shown in Table 1. These localities were chosen because in each one there were amphibian species with some degree of threat. At each site, a search and visual encounter technique was employed [67] to locate amphibian species. The amphibians were collected individually in plastic bags to avoid cross-contamination and contact with the skin of the collector. Once in the laboratory, each individual was handled with sterile gloves. For *Bd* isolation, 14 non-threatened amphibian species were collected. From each species, a maximum of 20 adults were collected and transported to the work station. Dead or visibly sick individuals of any species were also collected. *Bd*-positive animals and animals visibly suffering from chytridiomycosis were euthanized, and later deposited in the Zoology Museum of the University of Costa Rica.

Pathogen detection and isolation

Distal sections of the amphibian hind limb digits were cut and skin pieces were observed for characteristic *Bd* sporangia. Those *Bd*-infected individuals were transported to the University of Costa Rica taking proper biosafety precautions. The procedure to isolate *Bd* was performed in accordance with the protocols established by Longcore *et al.* [68–70]. Animals positive for the pathogen were sacrificed by pithing [71] or with MS-222 (tricaine methanesulfonate) for larger individuals. Skin tissues between the posterior digits and ventral skin were removed with sterile needles or scissors. A small section of infected tissue was cleaned of contaminants and cultured on TGH agar (0.8% tryptone and 0.2% hydrolysed gelatin) with 0.2 mg ml^{-1} penicillin-G and 0.3 mg ml^{-1} streptomycin sulphate. Upon observing colony formation, agar sections were transferred to 25 cm^2 cell culture flasks containing 13 ml of liquid TGH. Cell culture flasks were maintained at 23 °C. We cryopreserved replicate cultures of all isolates at −80 °C in TGH broth with cryoprotectant solution [70] and deposited them in the microbial culture collection of the Research Center in Microscopic Structures (CIEMic) located at the University of Costa Rica, until their reactivation.

Genetic and morphological characterization of *Bd*

The preserved *Bd* isolates were reactivated according to the protocol of Longcore [69]. For DNA extraction, a sample of

Table 2. Loci identified for Costa Rican *Bd* isolates and their primer sequences

Locus	Genomic contig (SC) and position*	Alleles	Primer sequence	Reference
8009×2	SC 1: 0.64 Mbp	4	F: 5′-TCGTGAAGAGCTTGGAAAGTCG-3′ R: 5′-AGTTCTGTCGTCAATGCTGTAGGG-3′	[109]
*Bd*C5	SC 1: 1.45 Mbp	3	F: 5′-TAATAGCGCCGACCGAACTA-3′ R: 5′-ATGCCAAACCATGAGCAAAT-3′	[110]
*Bd*SC3.1	SC 3: 0.17 Mbp	3	F: 5′-CAGTGACTTGCATCCACGAG-3′ R: 5′-AATCGCTTCAACCAAACTGG-3′	[22]
*Bd*SC4.16	SC 4: 1.64 Mbp	3	F: 5′-TCAACTGGCTTTGAGCACAC-3′ R: 5′-ATAGAGCATGCAGATCGCTTT-3′	[22]
R6046	SC 5: 1.22 Mbp	2	F: 5′-CTATCTGCGCTCCCGTGTCAA-3′ R: 5′-AGGGCTGCAACAACTGGATTT-3′	[111]
*Bd*SC6.15	SC 6: 1.51 Mbp	4	F: 5′-GACGATAAAACGACAACAATCG-3′ R: 5′-CCCTTTTTAGGTTGGCTTGC-3′	[22]
*Bd*SC7.6	SC 7: 0.66 Mbp	2	F: 5′-TGTGCCCGTGTTTTTGATTA-3′ R: 5′-GTTACAACCTCCCGCTCGTA-3′	[22]
8702×2	SC 16: 0.27 Mbp	2	F 5′-ACCAACTATAACATCATCAAG-3′ R 5′-GAATATGGCATGGGAGAAGTAGCC-3′	James *et al.* 2009

*The positions are based on the assembly of the genome of strain JEL423 carried out by the Broad Institute (version 17 January 2007), and deposited in the Bioproject PRJNA13653 of the NCBI. The markers correspond to genes that code for hypothetical proteins.

a solid culture in TGH medium containing the pathogen was taken with a sterile applicator following 3–4 days of growth. DNA extraction was performed according to the protocol of Zhang *et al.* [72]. The crude extract of genomic DNA was stored at −20 °C. PCRs were performed using different markers as indicated below. Reaction mixes of 50 µl were prepared containing 5 µl 10× Dream Taq Buffer, 2.5 µl of PCR primers at a concentration of 20 µM, 0.25 µl of taq polymerase 'DreamTaq TM DNA Polymerase' (Thermo Fisher Scientific), 1 µl of the four dNTPs at a concentration of 10 mM, 2 µl of genomic DNA, and 0.1 µM of BSA as an additive. The volume was completed by adding PCR-grade water. The amplification reaction was performed in a 2720 Thermal Cycler (Applied Biosystems), using a programme consisting of an initial denaturation at 94 °C for 3 min, followed by 35 cycles of: 94 °C for 60 s, 50 °C for 30 s and 72 °C for 1 min, and a final extension phase at 72 °C for 7 min. PCR products were visualized by electrophoresis with 1% agarose gels stained with GelRed (Biotium). We sequenced eight polymorphic multilocus (MLST) loci (described in Table 2) using Sanger sequencing (Macrogen). Because *Bd* is diploid and the amplified genes have several alleles, the heterogeneity of the sequences was checked with the QSVanalyzer software (dna.leeds.ac.uk/qsv/). Sequences were merged to obtain a consensus sequence and a genotype was assigned to each fungal isolation through the identification of SNPs employing Gene Studio software (GeneStudio). Each allele was ordered and analysed together through a Nexus file (Material S2, available in the online version of this article). For each sequenced marker, a letter was assigned according to the identified allele, so that the program associates a letter to the corresponding sequence of each allele. Therefore, each genotype was assigned a sequence of letters according to its alleles [71]. A dendrogram was constructed with the neighbour-joining algorithm [73] with PAUP v4.0b10 software [74], using sequences of previously published genotypes after clone correction (removal of identical genotypes within a geographical population to account for non-independent sampling; Table S1). The values for each sequence of the neighbour-joining dendrogram were inferred by a bootstrap on 10000 replicates.

We compared sporangia and spore size of each *Bd* isolate from a liquid culture in TGH medium grown at 23 °C for 7 days [19, 24]. For this, 60 photographs of sporangia and 60 photographs of spores from each isolate were taken with a light microscope employing a 1000× magnification. Diameters were measured using DP Manager 3.3.1 software (Olympus Corporation 2001–2007). A one-way ANOVA and a subsequent Tukey test were performed with the vegan package in R Studio software v1.0.136, and plotted with the ggplot2 v.2.2.1 package. Additionally, the number of germinative tubes for each isolate was recorded using the images taken from sporangia 7 days after inoculation and compared using ANOVA tests.

To determine the optimal growth temperature of each isolate, 50 µl of a 5 day *Bd* culture in TGH broth was transferred to 96-well plates, along with 50 µl of TGH broth. Plates were incubated at different temperatures (5, 15, 18, 20, 23, 25 and 30 °C) for 7 days. The absorbance at 492 nm of each plate was recorded daily with an ELISA Microplate Reader (VMax Kinetic ELISA Microplate Reader). To compare the growth of each isolate at different temperatures (growth value), we subtracted the absorbance value of day 7 minus the absorbance at day 1 for each replicate. Twenty-four replicates were performed per isolate and growth values obtained were compared by one-way ANOVA and plotted with the ggplot2 package in RStudio as described above.

Table 3. Species of Costa Rican amphibians used for the isolation of epidermal bacteria and number of bacterial isolates obtained from each species

Location	Species	*N*	Preserved bacteria	Preserved actinomycetes
Río Murciélago	*Craugastor ranoides*	20	18	2
Cerro Chompipe	*Incilius holdridgei*	8	6	1
Santo Domingo	*Agalychnis annae*	20	31	1
Rancho Chutas	*Lithobates vibicarius*	20	12	3
Las Cruces	*Ptychohyla legleri*	20	30	0
Veragua	*Agalychnis lemur*	20	24	8
Punta Banco	*Craugastor taurus*	20	6	6
Santa Elena	*Duellmanohyla rufioculis*	20	10	1
	Espadarana prosoblepon	2	4	0
	Hyalinobatrachium colymbiphyllum	1	1	1
La Selva	*Oophaga pumilio*	20	21	11
	Craugastor bransfordii	20	4	3
Total		**191**	**167**	**37**

Bacterial isolation

To obtain culturable bacteria from amphibian skin, we sampled 191 individuals of 12 frog species from different localities in the country (Tables 1 and 3). For each individual, weight, length and sex were recorded, and animals were released at the location of capture after skin microbe sampling. Wearing a clean pair of nitrile gloves, we washed the entire body of the individuals for approximately 7 s with sterile distilled water. The whole skin surface was rubbed between 10 and 15 times with sterile dry swabs (MW100, Advantage Bundling SP; LLC) after which swabs were rubbed on a Petri dish with R2A agar [75], rotating the swab over the medium throughout the process. When separate colonies were observed, they were purified by taking a section of the colony with a sterile loop and inoculating it into a new Petri dish with LB agar with antifungals (Material S1). The plates were checked daily to detect the presence of colonies. Purified isolates were preserved in cryovials with LB and 40% glycerol. Preservation was performed in duplicate, submerging each cryovial in liquid nitrogen followed by storage at −80 °C.

Because actinomycetes are recognized antibiotic producers [76], we also employed chitin agar for their specific isolation [77]. After a second washing of the individual, the procedure above was repeated, inoculating a new swab with sample on a Petri dish with chitin agar. The colonies from the culture medium were later transferred to YMEA medium [78] with antibiotics (Material S1). Purified isolates were preserved in liquid YMEA medium with 20% glycerol, submerged in liquid nitrogen and stored at −80 °C.

Bd-Growth inhibition assays

Bioassays to test the inhibitory activity of bacteria isolated from amphibian skin were carried out first against a hypervirulent strain of *Bd* from Costa Rica, isolate JEL732 [79], classified as *Bd*-GPL-2 [21]. Subsequently, only those bacteria that showed inhibitory activity against this isolate were screened against the other *Bd* isolates obtained from different regions in Costa Rica. The inhibition bioassays were performed in 96-well plates according to the protocol of Bell *et al*. [80]. A bacterial cell-free supernatant (CFS) obtained from a 48 h liquid culture of each bacterial isolate was employed for testing *Bd* inhibition against a suspension of 2×10^6 *Bd* zoospores from a 72 h solid culture of less than three passages. Bioassays were performed in 96-well plates, placing 50 µl of TGL medium + 50 µl of *Bd* zoospore suspension for negative controls and 100 µl of TGH medium and 50 µl of TGL medium + 50 µl of *Bd* zoospores suspension killed by heat (60 °C for 60 min) for positive controls. In the experimental wells, 50 µl of *Bd* zoospore suspension + 50 µl of the CFS were placed in five replicates. Assay plates were incubated at 23 °C for 8 days, and growth was measured every day as the optical density (OD) at 492 nm on a spectrophotometer (VMax Kinetic ELISA Microplate Reader). Percentages of inhibition were calculated by dividing the corrected absorbance value for each sample on its maximum growth day by the corrected absorbance value of the positive control on its maximum growth day, according to the data adjustments shown in Bell *et al*. [80] and averaging the values of the five replicates for each *Bd*–bacteria assay. Finally, an ANOVA was performed to compare the percentages of inhibition of each bacteria against each *Bd* isolate.

Bacterial sequencing and identification

The bacterial isolates that showed inhibitory activity against *Bd* JEL732 were identified by sequencing their 16S rRNA gene. In the case of non-actinomycete bacteria, a direct colony PCR was performed. For actinomycetes, 1 mg of cells from a 2–3 day old culture were taken and DNA was extracted employing a phenol/chloroform protocol [81]. PCRs were

Table 4. Amphibian species analysed by tissue observation, number of individuals positive for the pathogen by tissue observations and isolates obtained from *Bd* in each locality

Site and species	Evaluated for *Bd*	*N* positive for *Bd*	Collected for the isolation	Isolations
El Tirol				
Lithobates taylori	13	10	13	1
Isthmohyla pseudopuma	1	0	0	0
Craugastor podiciferus	1	0	0	0
Santo Domingo				
Lithobates taylori	6	4	4	0
Lithobates warszewitschii	5	1	3	0
Guayacán				
Craugastor crassidigitus	5	0	2	0
Craugastor fitzingeri	2	0	2	0
Craugastor cf. *rearki*	1	0	0	0
Teratohyla spinosa	3	0	0	0
Lithobates warszewitschii	6	0	3	0
Leptodactylus savagei	1	1	1	0
Oophaga pumilio	1	0	1	0
Uvita				
Atelopus varius	3	3	3	*
Las Tablas				
Lithobates taylori	2	2	2	1
Espadarana prosoblepon	2	1	2	1
Craugastor fitzingeri	5	1	5	0
Craugastor crassidigitus	3	0	0	0
Craugastor gabbi	2	0	0	0
Las Cruces				
Craugastor fitzingeri	5	2	2	1
Craugastor crassidigitus	5	0	0	0
Espadarana prosoblepon	5	0	0	0
Lithobates warszewitschii	2	0	0	0
Pristimantis cruentus	2	0	0	0
Punta Banco				
Craugastor fitzingeri	10	0	0	0
Smilisca sila	10	0	5	0
Total	**101**	**25**	**48**	**4**

*No isolation, but DNA was obtained from a specimen for genetic analysis.

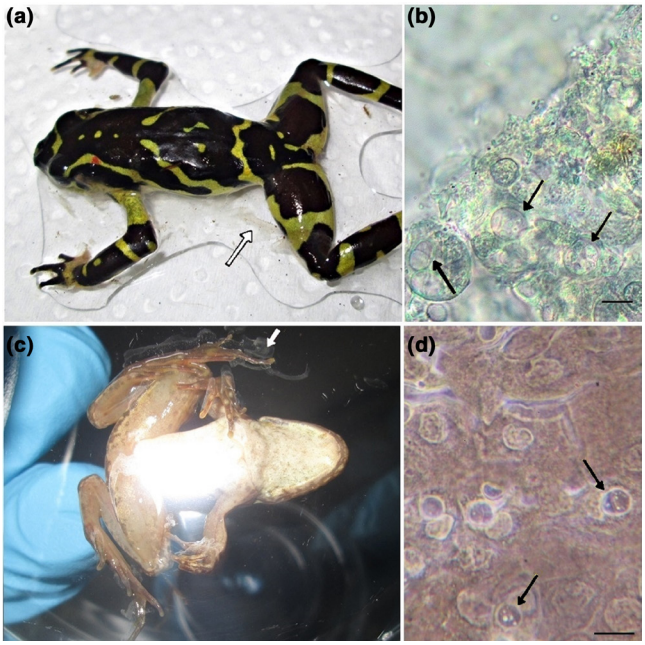

Fig. 1. Costa Rican amphibians with symptoms of chytridiomycosis. (a) *Atelopus varius* found moribund, (b) samples of loose tissue of *A. varius*, (c) *Lithobates taylori* and (d) tissue samples of *L. taylori*. White arrows indicate loose tissue, and black arrows indicate the presence of *Bd* sporangia. Scale bar: 10 µm

performed with the bacterial primers 27F and 1492R in a 2720 Thermal Cycler thermocycler (Applied Biosystems), using a programme consisting of initial denaturation at 95 °C for 5 min, followed by 30 cycles of: 94 °C for 45 s, 51 °C for 50 s and 72 °C for 120 s, and a final extension phase at 72 °C for 10 min. PCR products were visualized by electrophoresis with 1 % agarose gels stained with GelRed (Biotium) and then sent to Macrogen for cleaning and sequencing. The sequences obtained were edited and assembled with the GeneStudio program (GeneStudio). To determine the taxonomic identity of each bacterial isolate, the sequences were compared with both the nucleotide collection (nr/nt) of GenBank using the BLAST program from the NCBI website [82] and with type lineages in the RDP database (http://rdp.cme.msu.edu/).

RESULTS

Genetic and phenotypic characterization of *Bd* isolates from Costa Rica

A total of 101 individuals belonging to 15 amphibian species were evaluated for the presence of *Bd*, resulting in detection of characteristic sporangia in 25 individuals from six different species (Table 4). Eight individuals showed clinical signs of chytridiomycosis: three *Atelopus varius* from Uvita, four *Lithobates taylori* from El Tirol and one *L. taylori* from Santo Domingo (Fig. 1). Four *Bd* isolates were obtained in pure culture: one from *L. taylori* collected at El Tirol (JGA01), one from *Craugastor fitzingeri* collected at Las Cruces Biological Station (JGA02), and two from Las Tablas Protected Zone, one from *Espadarana prosoblepon* (JGA03) and the other from *L. taylori* (JGA04). At the other sites, despite detecting *Bd* presence in several species, we could not isolate the pathogen due to plate contaminations or because they had very low numbers of sporangia; for example, a deceased individual of *Atelopus varius* from Uvita had high abundance of sporangia but high bacterial contamination did not allow adequate isolation. Even so, DNA was extracted for genetic analysis. Consequently, in the MLST we include six *Bd* from Costa Rica: four isolates, the genetic material from *Atelopus varius* at Uvita and the previously obtained isolate from La Selva Biological Station (JEL732) [79].

With the exception of 8702×2, all primers generated the expected PCR products. Six loci were homozygous while only the *Bd* SC7.6 locus showed heterozygosis between alleles 1 and 2. The result of the population genetics analysis is shown in a dendrogram constructed with the neighbour-joining method (Fig. 2, Material S2). The dendrogram shows that the six isolates from Costa Rica are within the global hypervirulent group *Bd*-GPL-2 and the closest genotypes are two isolates from Panama (PM1 and JEL423) and two from Brazil (CLIFT 035-042). Bootstrap values only show a statistically robust differentiation between the lineages of Brazil, the hypervirulent strain *Bd*-GPL, and between the groups within this hypervirulent strain. The results also suggest a possible separation within Costa Rica of genotypes corresponding to the central part (La Selva, El Tirol and Uvita) and the extreme south of the country (Las Tablas and Las Cruces as well as an isolate from Guabal, Panama) but genetics do not support a clear group separation.

We found significant differences in the diameter of the sporangia and the spores among the four *Bd* isolates obtained in pure culture (P=4.25e-06, P=1.15e-09) (Fig. 3). Additionally, a significant difference was observed between the maximum number of germinative tubes (P=8.08e-11) with Tukey tests showing that the JGA02 isolate differed significantly from the others (P=4.60e-06 with JEL732; P=3.00e-07 with JGA01; P=1.00e-07 with JGA03). Isolate JGA02 had one to three germ tubes while other isolates rarely exceeded two germ tubes and in many instances only had a spore releasing opening (Fig. 4). For optimal *Bd* growth we tested three isolates, because JEL732 did not grow well at different temperatures. Optimal growth of each fungal isolate varied according to temperature (JGA01 F=71.04, P=2.20e-16; JGA02 F=75.55, P=2.20e-16; JGA03 F=119.06, P=2.2e-16). For isolates JGA01 and JGA03, highest growth was observed at 23 °C, whereas for JGA02 optimal growth was observed at 18 °C (Fig. 5).

Inhibition of Costa Rican *Bd* isolates by cutaneous bacteria

In total, 204 bacterial isolates were obtained from 191 individuals belonging to 12 different frog species (Table 3). We were able to test the anti-*Bd* potential of 185 of those isolates, while 19 isolates could not be evaluated due to contamination or inability to recover the strain after preservation. First,

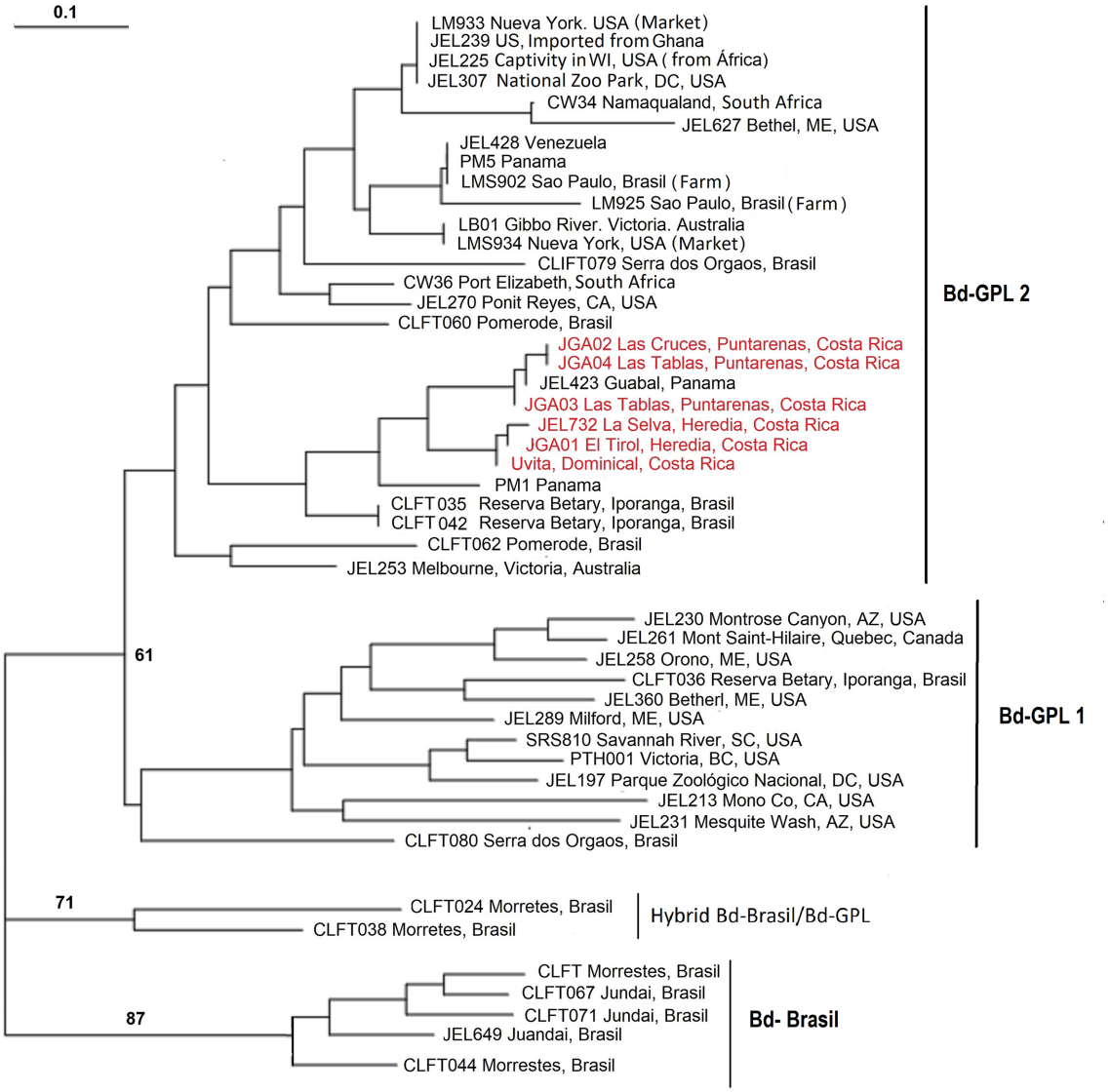

Fig. 2. Dendrogram showing the genetic relationships between *Bd* lineages based on typing by MLST. The codes in red represent the isolations from this study. The dendrogram was performed using seven genetic markers with the neighbour-joining method in the PAUP software package (with a total of 3006 bp). Bootstrap support values of 10000 replicas are shown for nodes with values greater than 50%. The isolates are considered GPL-1 if they are heterozygous or homozygous for allele 1 in the R6046 locus [22]. The scale bar represents average substitutions per nucleotide position

we assessed the antifungal activity of all the isolates against JEL732. Of the 185 isolates tested, 50 showed antifungal activity greater than the threshold of 63.5% proposed previously [80] (Table S2). In total, 16 different bacterial genera with inhibitory activity against JEL732 were identified: *Stenotrophomonas, Chryseobacterium, Serratia, Pseudomonas, Streptomyces, Bacillus, Enterobacter, Kocuria, Acinetobacter, Sphingobacterium, Staphylococcus, Klebsiella, Microbacterium, Paracoccus, Rhizobium* and *Pseudocitrobacter*.

Subsequently, these 50 bacterial isolates (plus nine that showed inhibition during the first 4–5 days but were contaminated by day 7) were tested against the other three Costa Rican *Bd* isolates. In total, 21 (nine genera), 11 (four genera) and nine (four genera) isolates showed inhibition against JGA01, JGA02 and JGA03, respectively (Table S3). In summary, 14 bacterial isolates belonging to *Stenotrophomonas, Streptomyces, Enterobacter, Pseudomonas* and *Klebsiella* had inhibition values greater than 63.5% for all four *Bd* isolates (Fig. 6). *Bd* inhibition differed between bacterial genera (F=4.5481, df=58, P<0.0001). A significant difference was found in the inhibition capacity of these 14 isolates against the four *Bd* isolates (ANOVA, P=9.3e-6, Fig. 7).

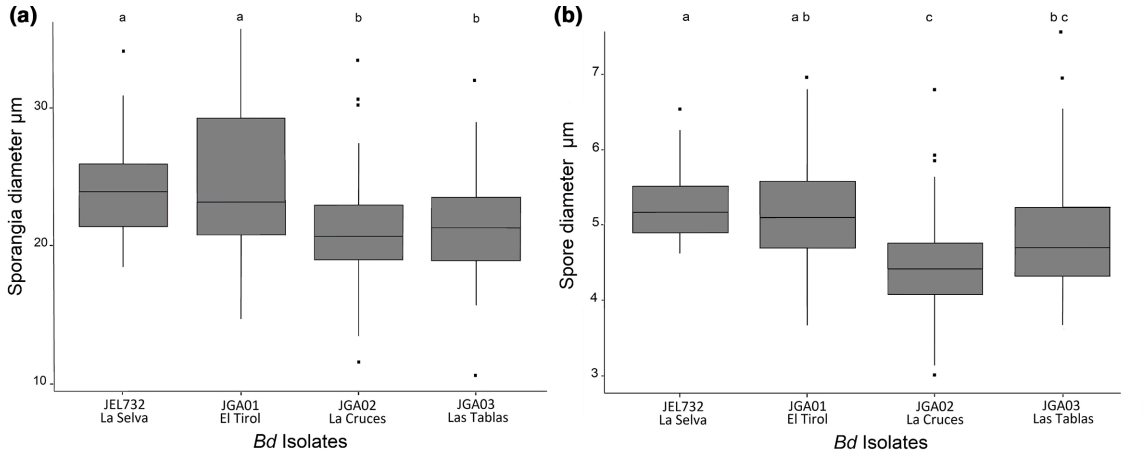

Fig. 3. Phenotypic characterization of *Bd* isolates from Costa Rica. (a) Sporangia diameter. (b) Spore diameter. The horizontal bars inside the boxes represent the average value and the shaded boxes show the standard error of the median. The samples were compared by ANOVA (letters above bars indicate groups that differ significantly).

DISCUSSION

Costa Rica is among the regions in the Americas where *Bd* has been intensively studied and frequently detected [21], but genotype data for *Bd* collected in this region are lacking although this topic has received much attention in recent years [73, 23, 83]. The results of the present study show that all the *Bd* isolates obtained in Costa Rica belong to the global *Bd*-GPL strain and are grouped within the *Bd*-GPL-2 clade. This *Bd* strain is considered highly virulent [22]. The wide geographical distribution of the *Bd*-GPL clade is related to repeated introduction events due to the global amphibian trade [23, 83]; and these repeated introduction events have been associated with declines in Central America and Australia [21]. *Bd* isolates from Costa Rica showed differences at the morphological level in sporangia size and number of germ tubes and differences in optimal growth temperature. The isolates from the central part of the country seem to have larger sporangia than southern isolates, which could be explained by a correlation between the size of the sporangia and the genotype [19, 24]. It has been suggested that mass death events both in Central America and in South America [84] were associated with the expansion of *Bd* from northeast to southeast [85, 86]. This movement towards the southeast could explain genetic separation in Costa Rica; however, the genetic data from this study are not sufficient to corroborate this geographical separation of the genotypes.

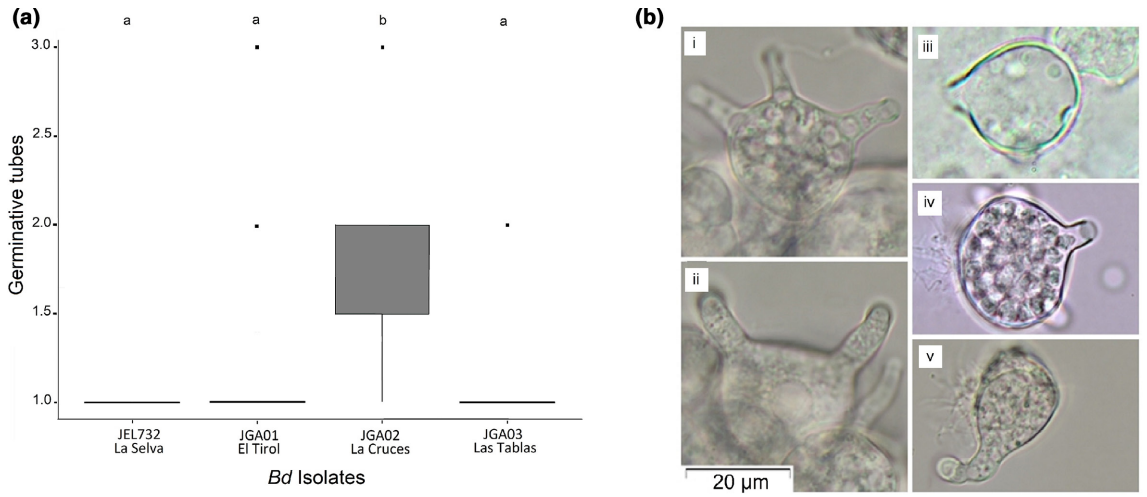

Fig. 4. Phenotypic characterization of germinative tubes from *Bd* isolates in Costa Rica. (a) Number of germinative tubes. The samples were compared by ANOVA (groups with significant difference are shown with the letters 'a' and 'b' on the bars). (b) Light microscopy (1000×): (i–ii) isolate JGA02 showing several germinative tubes, (iii) isolate JEL732 showing an opening in place of a germ tube, (iv) isolate JGA01 and (v) isolate JGA03 showing a single germ tube.

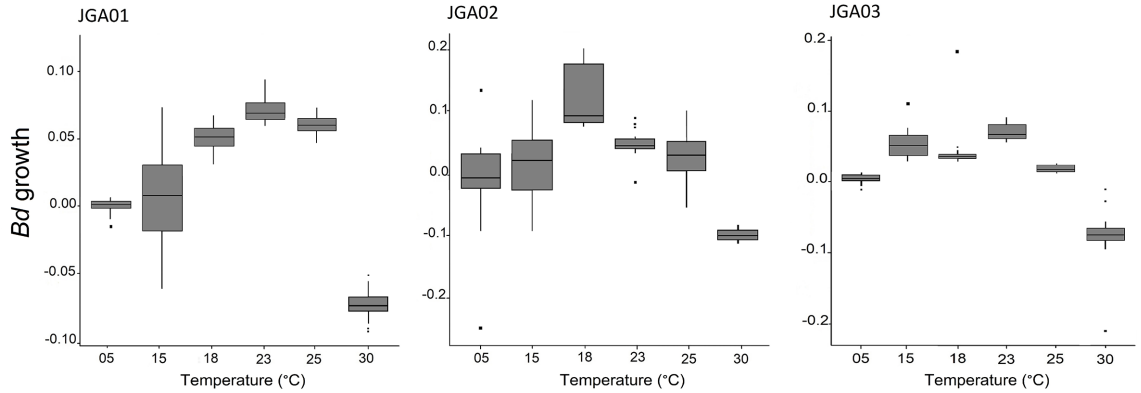

Fig. 5. Growth values of three *Bd* isolates at different temperatures. Growth data were obtained with the difference of the absorbance value at 492 nm between the first and seventh day of incubation. Horizontal lines within the boxes represent the average; shaded boxes represent the standard error of the median.

Isolate JGA02 showed a greater number of germinative tubes, which could imply a greater spore discharge capacity and therefore greater virulence; this relationship could be evaluated with infection trials. In addition to germinative tubes, this isolate was the only one that had an optimum growth temperature of 18 °C. These differences suggest a different phenotype for this isolate obtained from Las Cruces Biological Station. Phenotypic variations in different isolates of the same strain have been explained by the presence of adaptive processes due to the interaction between the pathogen, the

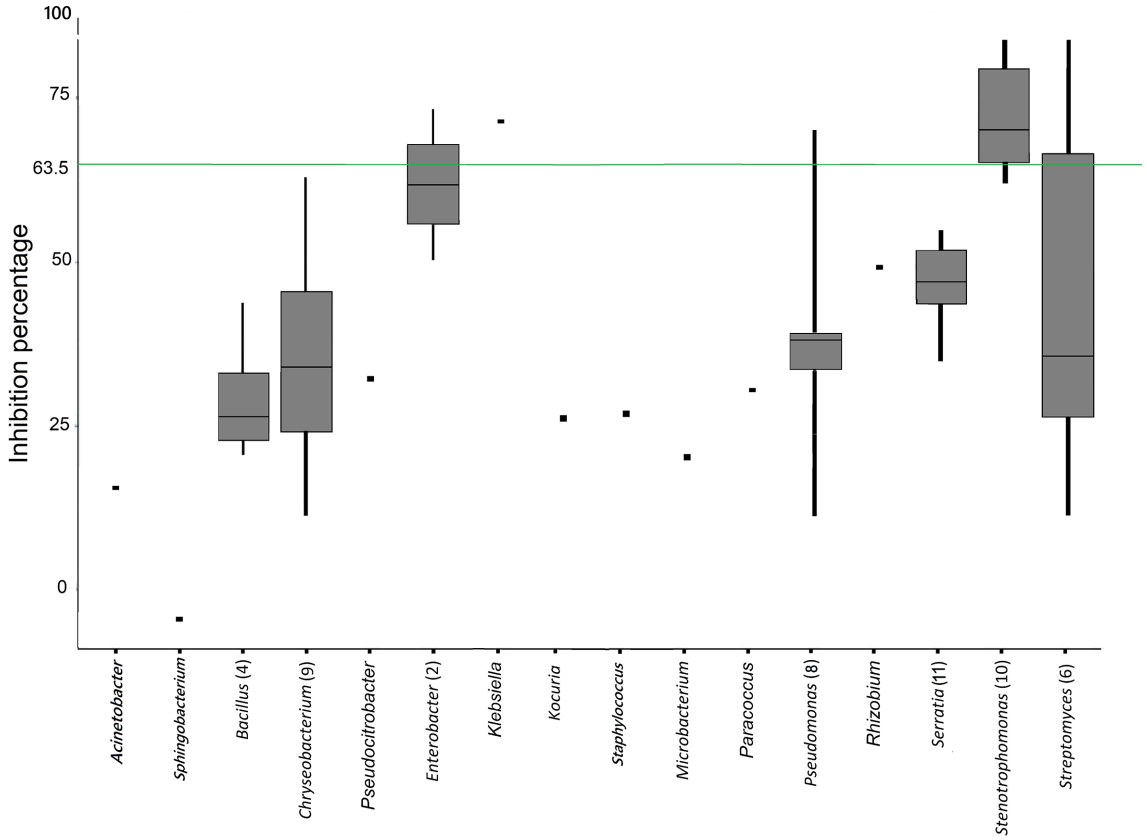

Fig. 6. Average bacterial inhibition against four *Bd* isolates from Costa Rica. Five bacterial genera had isolates that inhibited all fungal isolates (*Stenotrophomonas, Streptomyces, Enterobacter, Pseudomonas* and *Klebsiella*). The total number of isolates tested for each genus is shown in parentheses. Horizontal lines represent the average, shaded boxes the standard error of the median, and vertical lines the maximum and minimum value. The green line represents the limit of effective inhibition according to Bell *et al*. [80].

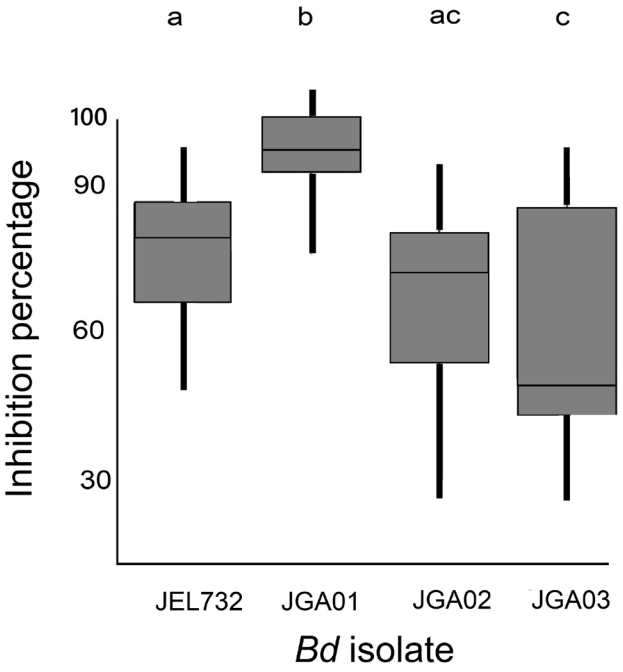

Fig. 7. Inhibition values of the 14 bacterial isolates with highest average inhibition against four Costa Rican *Bd* isolates. Horizontal lines within the boxes represent the average, and shaded boxes represent the standard error of the median. Groups which differ significantly are shown with a different letter above the bars.

host and the environment [25] and have also been related to variation in virulence [87]. The reason why this isolate may be different is not clear, so further studies are needed to clarify these morphological changes.

The presence of the *Bd*-GPL strain in the country is a latent threat to amphibians due to its virulent nature and its high prevalence in many locations [65]. It is also of concern that a possible recombination and hybridization event could increase its virulence [22, 73, 88]. More importantly, we cannot rule out the presence of native lineages, as *Bd* was detected in Costa Rica as early as 1964 [17] and our isolates were obtained from sites with reported declines. Thus, it is necessary to check whether the reports prior to the declines belong to the hypervirulent strain or not. It is also imperative to isolate more lineages of *Bd* from areas not associated with declines, in order to have a more complete picture of the genetic diversity of the pathogen in Costa Rica. However, we can eliminate the possibility that a non-virulent strain of *Bd* allows the persistence of these relict populations, at least for the four populations from which we isolated *Bd*. As recently suggested by Voyles *et al.* [89], host recoveries are not caused by pathogen attenuation, and its survival could be more related to environmental conditions and/or host responses.

To investigate the cutaneous bacteria as a possible host response, we did an initial bioassay against isolate JEL732. This revealed 16 bacterial genera that showed inhibitory activity against that isolate (mainly *Stenotrophomonas, Chryseobacterium, Pseudomonas, Serratia, Streptomyces* and *Bacillus*); the genera and values of inhibitory activity were similar to those reported in other studies [59, 90–96]. In contrast, when we ran a second series of bioassays against the fungal isolates JGA01, JGA02 and JGA03, many of the bacterial isolates catalogued previously as inhibitory did not have the same inhibitory capacity, suggesting a differential inhibition depending on the challenged *Bd* isolate. Inhibition differences among *Bd* lineages and bacterial isolates have been previously reported in Belize [97, 98] and Madagascar [99]. Only some bacterial species within each genus have anti-*Bd* capacities [99], and we observed in this study that not all isolates of the same bacterial species have the same inhibitory capacity.

Differential inhibition was observed in the genera *Chryseobacterium* and *Serratia*. Also, some colourless lineages of *Serratia marcescens* do not produce inhibition while those with red pigment do [96], suggesting that the production of prodigiosin may play a role in the inhibition of *Bd* [100, 101]. Although some bacteria genera show better inhibitory activity, it has been suggested that probiotic efficacy cannot be expected to be uniform throughout the genetic or phenotypic landscape of the pathogen [97]. In our experiments, 14 bacterial isolates showed values above the threshold established as inhibitory when tested against the four *Bd* isolates. Those isolates belong to the genera *Stenotrophomonas, Streptomyces, Enterobacter, Pseudomonas* and *Klebsiella*, and would be good candidates for continued research aimed at developing probiotics [64].

In microbiological environmental studies there is usually a gap between laboratory experiments and the functionality of these results in nature. This problem is sometimes due to the use of unsuitable culture media [102]. This study is the first one that uses a specific medium for actinomycetes (chitin agar) and obtained several isolates of the genus *Streptomyces* with promising inhibitory activity from five different species of amphibians in Costa Rica. The genus *Streptomyces* is usually not obtained with the widely used R2A medium [102, 103]. This genus is known for the amount of compounds with antibiotic activity that it can produce [76, 104], as well as for being a source of antifungal substances [105]. Bacteria of this genus have been used to combat diseases in plants and animals caused by microorganisms [106] and are a promising group in the search for compounds that prevent the growth of *Bd*. Therefore, we recommend the use of chitin agar medium in the search for more actinomycetes that can be tested as amphibian probiotic bacteria.

In this work we obtained a bacterial collection representative of several amphibian species in Costa Rica and were able to verify the presence of bacterial genera capable of inhibiting the growth of *Bd* in some surviving populations of threatened amphibians. Species such as *Agalychnis annae*, *Agalychnis lemur* and *Craugastor ranoides* survive in relict populations [33, 65] and the presence of a greater number of inhibitory bacterial genera could be part of the reason why they have survived chytridiomycosis. Although some bacterial species

have been shown to be very effective in the inhibition of *Bd* isolates from Costa Rica, they may not be effective when used against other *Bd* lineages [99], highlighting the importance of obtaining a variety of local bacterial isolates. While it is important to continue the genetic study of the pathogen, we also recommend studies that enhance our understanding on how host and environmental factors allow frog species to tolerate and survive the pathogen. Studies on *Bd* and chytridiomycosis are becoming more frequent and new technologies and protocols are developing rapidly. Recently, a form of genotyping from swab samples was described [107] and protocols for the isolation of the pathogen without the need to apply euthanasia to individuals are being developed [108]. Along with those techniques, it is necessary to develop new culture media and host research methodologies in order to maximize our understanding of the dynamics of the pathogen, while reducing the risk to amphibians when working with endangered species.

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Author contributions
S.M.W., J.K., G.A., J.G.A. and A.P.T. conceived and designed the experiments. J.G.A., S.M.W., I.Z. and C.M.C. performed the experiments. J.G.A., S.M.W. and A.P.T. analysed the data. S.M.W., I.Z., C.M.C. and A.P.T. contributed reagents, materials and/or analysis tools. J.G.A., S.M.W., I.Z. and A P.T. wrote the paper. S.M.W., J.K. and C.M.C. reviewed drafts of the paper.

Conflicts of interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical statement
Biodiversity access permits were granted by the Institutional Commission of Biodiversity of the University of Costa Rica (Resolution 014 and 044) and collecting permits were granted by the National System of Conservation Areas (Resolution of scientific research No. 003-2016-ACCVC-PI). The sacrificing of *Bd* positive animals and animals visibly ill from chytridiomycosis was performed in accordance with the procedures established by the Institutional Committee for the Care and Use of Animals of the University of Costa Rica (CICUA Permit: No. 029-15).

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