Caracterización De La Comunidad Bacteriana Residente En La Superficie Epidérmica De *Bolitoglossa nicefori* (Caudata: Plethodontidae) De La Cordillera Oriental De Colombia.

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Dedicatoria

Dedico este trabajo principalmente a mis padres, quienes han sido siempre el motor de mi vida y me han brindado todos los medios necesarios para perseguir mis sueños, a pesar de las adversidades. Por su amor incondicional, les estoy infinitamente agradecido, me llena de orgullo ser su hijo.

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PALABRAS CLAVE: BOLITOGLOSSA, SALAMANDRA, MICROBIOTA, DIVERSIDAD ALFA, METAGENOMA.

El mucosoma de anfibios es considerado un microecosistema selectivo que interactúa con su alrededor; éste es habitado por una comunidad microbiana diversa que ofrece protección al huésped y se beneficia de él. *Bolitoglossa nicefori* es una salamandra terrestre endémica de la Cordillera Oriental de los Andes. En este estudio examinamos la estructura de la comunidad bacteriana en la piel de individuos de *B. nicefori*. Evaluamos el efecto de la precipitación en la composición y abundancia relativa de las bacterias residentes en la piel de esta especie usando Illumina MiSeq. Adicionalmente, caracterizamos la fracción de bacterias cultivables de la piel de estos individuos. Evidenciamos que la microbiota epidérmica en individuos sanos exhibe una alta diversidad alfa, dominada por dos phyla principales, Proteobacteria y Bacteroidetes. Una estructura similar se observó en la fracción de bacterias cultivables. Hasta ahora, poco se sabe acerca de la respuesta de la microbiota frente a la influencia de factores abióticos. Nuestros resultados revelaron que el incremento en la precipitación tuvo un efecto positivo en la diversidad alfa, favoreciendo la colonización de nuevos filotipos de baja abundancia. A pesar de esto, la estructura de la comunidad bacteriana se mantuvo estable. Nuestros hallazgos en *B. nicefori* apoyan la idea de que los anfibios y la microbiota cutánea residente mantienen una fuerte relación simbiótica, que, a pesar de estar influenciada por el ambiente, es resiliente a factores abióticos como cambios en los niveles de humedad.

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* Trabajo de grado.

** Escuela de Biología, Facultad de Ciencias, UIS. Director: Jorge Hernández Torres, Ph.D.

Abstract

TITLE: CHARACTERIZATION OF THE CUTANEOUS RESIDENT BACTERIAL COMMUNITY IN *Bolitoglossa nicefori* (CAUDATA: PLETHODONTIDAE) FROM THE EASTERN ANDES OF COLOMBIA*

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KEYWORDS: BOLITOGLOSSA, SALAMANDER, AMPHIBIAN SKIN MICROBIOTA, ALPHA DIVERSITY, METAGENOME.

The amphibian skin mucosome is considered a selective microecosystem that interacts with its surrounding environment; it is inhabited by a diverse microbial community that protects the host and benefits from it. *Bolitoglossa nicefori* is a lungless, terrestrial salamander endemic of the Eastern Colombian Andes. We conducted a study of the skin-associated bacteria in a population of *B. nicefori*. We examined the bacterial community structure in the wettest and driest months to assess whether changes in precipitation affected the composition and relative abundance of bacterial epibionts. In parallel, we performed a microbial culturomics approach, to characterize the culturable bacteria. We evidenced that the skin microbiota in individuals of this species exhibits an overall high alpha diversity dominated by two major phyla, Proteobacteria and Bacteroidetes. A similar structure was observed in the culturable fraction. Hitherto, little is known about the response of the amphibian skin microbiota to the influence of abiotic factors. Our results show that increased rainfall levels had a positive effect on alpha diversity, favouring colonization of new low-abundance phylotypes. Despite this, the structure of the bacterial core community remained stable. Our findings on *B. nicefori* support the hypothesis that amphibians and the resident cutaneous microbiota share a strong symbiotic relationship and that, even though it is influenced by the environment, the structure of the core community is resilient to abiotic factors like changes in humidity levels.

* Trabajo de grado.

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Introduction

The salamanders of the genus *Bolitoglossa* (Plethodontidae) exhibit terrestrial habits and, unlike other amphibians, they rely almost exclusively on cutaneous respiration given their lack of lungs. For this reason, plethodontid salamanders usually occupy heavily moist microhabitats to avoid skin desiccation and facilitate gas exchange (Whitford & Hutchison, 1965; Ruben & Boucot, 1989; Winters & Gifford, 2013). It has been shown that high humidity conditions in the skin promote the proliferation of a diverse array of symbiont and pathogenic bacteria (Grice & Segre, 2011). The microhabitat of *B. nicefori* is highly moist and rich in decomposing organic matter. This terrestrial salamander spends most of its time within the rhizospheric soil, decaying wood, leaf litter, and the surrounding vegetation. As a result, individuals of this species are constantly in contact with different substrates that are rich in microorganisms (Lim et al. 2005). Indeed, amphibians obtain their skin bacteria from environmental sources (e.g., soil, leaflitter, vegetation, water bodies), which supply a highly diverse microbial reservoir and play an important role in modulating the host skin microbiota (Loudon et al. 2014; Bird et al. 2018). Most environmental bacteria transiently colonize the skin of amphibians, whereas only a fraction of this pool of microorganisms successfully becomes part of the resident community (Culp et al. 2007; Belden et al 2015; Sanchez et al. 2016; Bletz et al. 2017). The association between the core community and the host is strong enough that some bacteria can survive even in the absence of an environmental reservoir (Loudon et al. 2014; Flechas et al. 2017). Despite this, it has been shown that the amphibian skin microbiota typically differs from its surrounding environment, having a lower alpha diversity and a more uneven distribution of bacterial phylotypes (Bletz et al. 2017; Prado-Irwin et al. 2017; Bates et al. 2018; Bird et al. 2018). For instance, while Gammaproteobacteria is broadly abundant in

amphibian skin communities, small and large-scale studies show that environmental substrates, specially soil, are mainly dominated by Alpha and Betaproteobacteria (Bletz et al. 2017; Delgado-Baquerizo et al. 2018).

Several extrinsic and intrinsic factors contribute to the structuring and regulation of the amphibian skin microbial community. In the first place, amphibians are armed with an array of mechanical and chemical defences including the host's immune system, skin sloughing, and the production of antimicrobial peptides and other glandular secretions of different chemical composition that select and control the skin microbial load (Becker & Harris, 2010; Kueneman et al. 2013; Meng et al. 2013; Woodhams et al. 2014; Longo et al. 2015; Muletz-Wolz et al. 2018). These aspects can vary among species, life history stages, sex and even body parts (Sanchez et al. 2016). However, other studies have found insignificant variation in some of these traits (Prado-Irwin et al. 2017). Despite the host's defence mechanisms, some bacterial phylotypes are adapted to live within the amphibian mucosome in a symbiotic mutualistic relationship (Loudon et al. 2014; Woodhams et al. 2014). Interactions amongst resident and transient bacteria are also important in shaping the amphibian skin microbiota by means of secretory compounds that can modify the microenvironment and be lethal to other microorganisms or by reducing colonization sites (Becker & Harris, 2010; Loudon et al. 2014; Woodhams et al. 2014; Longo et al. 2015; Muletz-Wolz et al. 2018). In fact, amphibian skin symbionts comprise the first line of defence against colonization of pathogenic fungi like *Mariannaea* sp. and *Batrachochytrium dendrobatidis* (Banning et al. 2008; Harris et al. 2009; Becker & Harris, 2010; Woodhams et al. 2015). In this sense, factors that can have a negative impact on the composition of the bacterial community in amphibians, climate change and the

use of pesticides, could dramatically alter their susceptibility to environmental pathogens (Harris et al. 2009). On the other hand, aspects like geographic location, elevation, habitat type, host ecomorphology, microhabitat use and environmental factors (e.g., temperature, humidity) have been found to strongly influence the diversity of amphibian skin microbial epibionts (Longo et al. 2015; Muletz-Wolz et al. 2018; Bletz et al. 2017; Bletz et al. 2017b; Prado-Irwin et al. 2017; Bird et al. 2018; Catenazzi et al. 2018). In this sense, the degradation of the habitat or microhabitat of a species can have direct ecological consequences on the alpha and beta-diversity of environmental reservoirs of bacteria and consequently affect the amphibian host skin microbiota (Harris et al. 2009; Lan et al. 2017; Zhou et al. 2017). All these factors may act individually or in combination to produce the uniquely selected set of dominant bacterial phylotypes observed in the cutaneous bacterial community structure of amphibians. We characterized the skin microbial community in a population of the salamander *Bolitoglossa nicefori* from the Eastern Andes of Colombia. In addition, we explored whether different precipitation levels affected the stability of the bacterial community structure using a metagenomics approach.

1. Objectives

1.1 Main objective

- characterize the community of resident skin-associated bacteria in a population of *Bolitoglossa nicefori* from the Eastern Colombian Andes, Santander department.

1.2 Specific objectives

- Collect swab samples from individuals of *Bolitoglossa nicefori* for the isolation of culturable bacteria and the extraction of metagenomic DNA.
- Identify the isolated bacteria through sequencing of the 16S rRNA gene as well as their morphological characteristics.
- Describe the whole bacterial community resident in the skin of *Bolitoglossa nicefori* in the dry and wet seasons through next generation sequencing.
- Detect the presence of the pathogenic fungi *Batrachochytrium dendrobatidis* and *B. salamandrivorans* in the study population.

2. Methods

2.1 Study site and sampling

The study site is located on the western slope of the Colombian Eastern Andes, Santander department, municipality of Tona, vereda Vegas del Quemado (Figure 1). The area is part of a local forest reserve that is no longer frequented by people. The ambient temperature does not vary greatly along the year and, the annual rainfall pattern shows defined peaks of highest and lowest precipitation events (Figure 2). Fieldwork consisted of four survey expeditions during early March, June, October and late December 2017. Night surveys were performed along a defined 200 m long transect covering an altitudinal range from 1,792 to 2,034 m of elevation. Salamanders were handled with new nitrile gloves for each specimen to avoid cross contamination (Appendix 2) and because nitrile gloves have a fungicidal effect on *Batrachochytrium dendrobatidis* (Mendez et al., 2008). Captured individuals included both sexes and different life-history stages (i.e., neonates, juveniles, adults), inferred by body size (Ortega et al., 2009). Environmental records of monthly mean precipitation, maximum and minimum temperature were obtained from the Instituto de Hidrología, Meteorología y Estudios Ambientales (IDEAM) and the WorldClim database (Fick & Hijmans, 2017). In addition, ambient temperature and relative humidity were recorded with a weather station, while skin and substrate temperature were measured using a digital laser infrared thermometer. The environmental data were adopted for bacterial culture. Immediately after capture, each specimen was rinsed two times with autoclaved distilled water to wash out dirt and transient bacteria, leaving only the resident microbiota. Our washing protocol was similar to Walker et al. (2015) but with several modifications adapting to the fieldwork conditions and the characteristics of the study species. We only used one pair of gloves and performed two baths per individual. Instead of petri dishes, specimens were placed inside sterile Whirl-Pak® bags or UV-

irradiated Ziploc® bags and swabbed twice with 5 to 10 strokes on the costal grooves and along the dorsal/ventral surfaces of head, legs, feet and tail using Puritan® HydraFlock® sterile flocked swabs (Harry et al. 2013). After swabbing, the salamanders were released back where they were captured. The samples were transported with ice gel packs kept at 4-8 ºC. Research permits were granted by Autoridad Nacional de Licencias Ambientales (ANLA, Resolución 0047 and 0260). This project was endorsed by the Comité de Ética en Investigación Científica (CEINCI) of Universidad Industrial de Santander.

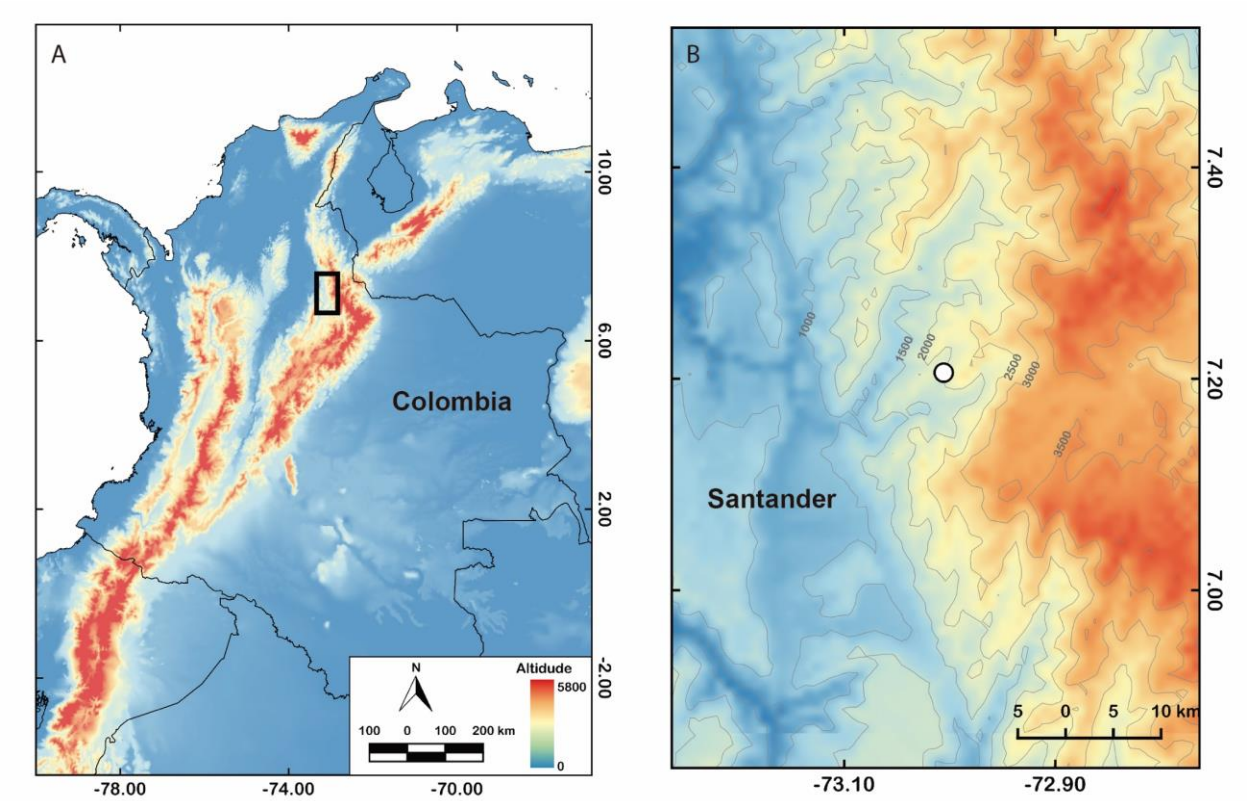

Figure 1. Map of Colombia indicating the location and altitude of the sampling site where the study species *Bolitoglossa nicefori* was found. Vereda Vegas del Quemado, municipality of Tona, Santander department, 2,000 m asl.

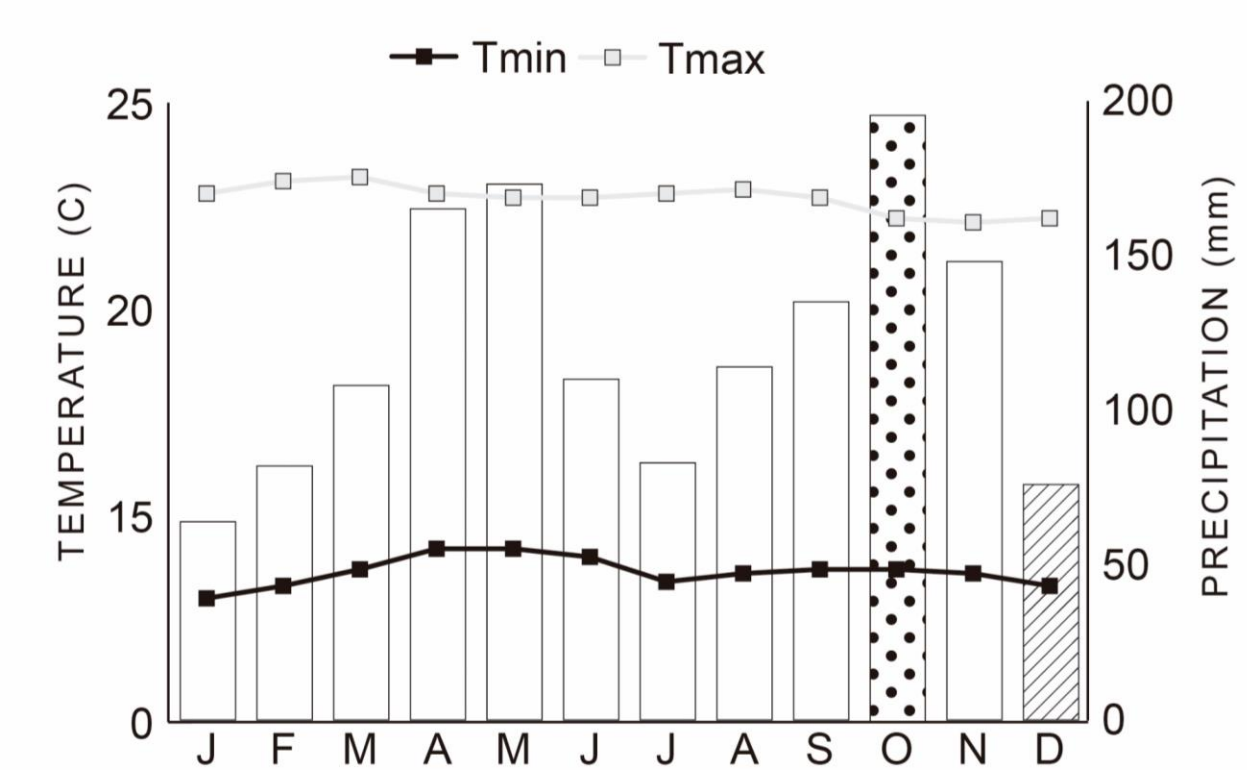

Figure 2. Monthly temperature and precipitation records of the study site. The wettest month is indicated with black points pattern. The driest month is indicated with oblique lines pattern.

2.2 Isolation of skin bacteria

To amplify the number of bacterial cells in the sample, 17 swabs were submerged in 3 mL Reasoner's 2A (R2A) broth (HiMedia®) shortly after swabbing and left incubating in a sterile test tube with screw cap for 3 days at the study site. Once in the lab, total cultures were cryopreserved at -80 ºC in R2A medium in individual cryogenic tubes with 30% glycerol as cryoprotectant. To maximize the number of bacterial isolates, 50 µL aliquots of total cultures and serial dilutions (1:10, 1:100, 1:1000) were plated on four different media: R2A (Reasoner's 2A Agar), CYC (Czapek-Dox Yeast Extract Casamino Acids), TSA (Tryptone Soya Agar) and LB (Luria Bertani Agar), as detailed in appendix 1. In addition, some swabs were stroked directly onto R2A, CYC, TSA and LB agar plates at the study site. Plate cultures were incubated for 14 days to allow growth

of low-abundance and slow-growing bacteria (Reasoner and Geldreich, 1985; Walke et al. 2015). Approximately 5% of each lot of agar plates and culture tubes were destined for environmental and quality controls to guarantee the sterility of the workplace and the medium. Similarly, during field sampling, some sterile swabs were submerged in R2A to discard possible airborne contamination of the culture medium. For the isolation of pure bacterial strains, colony forming units were picked based on their unique morphology (i.e., form, pigmentation, elevation and margin) on the different culture media (Bergys et al. 1974) and plated on separate agar plates. Furthermore, to aid in bacterial characterization and discrimination, fluorescence and iridescence were examined by exposing 48 h old plates to UV light at 365 and 395 nm and epi-illumination/transillumination with an oblique light source (white led light) at different illumination angles. Finally, a single colony from each isolated strain was cultured in 6 mL R2A broth for 3 d at 20 ºC. Pure cultures were cryopreserved in 30% glycerol and stored in 1.8 mL aliquots at -80 ºC.

2.3 DNA Isolation, amplification and sequencing

Whole genomic DNA was extracted from swabs, as well as from enrichment cultures and isolated strains. DNA isolation was performed using QIAamp DNA Mini Kit (Qiagen®, #51304) following the manufacturer's protocols for bacteria. The PCR mix consisted of 25 µl reactions containing 1 µl (≈ 30-50 ng/µl) genomic DNA, 12.5 µl of 2X TopTaq Master Mix (Qiagen®, #200403) consisting of 1.25 U TopTaq DNA Polymerase, 200 µM dNTPs, 1.5 mM $MgCl_2$ and 1X PCR Buffer, and finally 0.5 µl (0.2 µM) of each primer. The PCR cycling conditions according to the authors listed in table 1. The quality of PCR amplicons was verified on electrophoresis gels, then purified and sequenced from both strands with capillary electrophoresis. The sequence of the oligonucleotides used in this study are detailed in table 1.

2.4 Cultured samples

The molecular classification of bacterial isolates was carried out by the analysis of the V4 (~266 bp, partial length) and V1-V9 (~1400 bp, full-length) regions of the 16S rRNA gene. The V4 hypervariable region was selected for its superior performance over other regions of the 16S rRNA gene for bacterial taxonomic classification (Mizrahi-Man et al. 2013). We amplified the V1-V9 regions in cases where amplification of the V4 region failed or the classification was ambiguous. Amplification of the V4 gene region was done using the degenerated primers 515FB (Parada et al. 2016) and 806RB (Apprill et al. 2015), following the standard protocol of the Earth Microbiome Project (Thompson et al. 2017). The V1-V9 gene regions were amplified by using primers 27F/1492R (Lane, 1991). To discriminate between strains assigned to the genus *Pseudomonas* by the 16S rRNA gene, we amplified the *rpoD* protein-encoding gene (~736 bp) using primers PseG30F/PseG970R (Mulet et al. 2009).

2.5 Metagenomics analysis

PCR amplification of the 16S rRNA gene for metagenomic analyses was carried out using primers Bakt341F/Bakt805R (Klindworth et al. 2013), which target the V3-V4 gene regions (~465 bp). For the metagenomic analysis, samples from each month were processed together in one batch. The meta-amplicon library was constructed on an Illumina MiSeq platform by Macrogen Inc.

2.6 Detection of pathogenic fungi

For the selective detection of *Batrachochytrium dendrobatidis* and *B. salamandrivorans* the ITS1 - 5.8S - ITS2 genes were amplified using primers Bd1a/Bd2a (Annis et al. 2004) and STerF/STerR (Martel et al. 2013), respectively.

Table 1. List of primers used in this study.

Gene	Primer	Sequence (5' to 3')	Fragment size	Gene region	Reference
16S rRNA	27F	AGA GTT TGA TCM TGG CTC AG	1400	V1-V9	Lane, 1991
	1492R	CGG TTA CCT TGT TAC GAC TT	1400	V1-V9	
	Bakt341F	CCT ACG GGN GGC WGC AG	465	V3-V4	Klindworth et al. 2013
	Bakt805R	GAC TAC HVG GGT ATC TAA TCC	465	V3-V4	
	515FB	GTG YCA GCM GCC GCG GTA A	390	V4	Parada et al. 2016
	806RB	GGA CTA CNV GGG TWT CTA AT	390	V4	Apprill et al. 2015
rpoD	PseG30F	ATY GAA ATC GCC AAR CG	736		Mulet et al. 2009
	PseG970R	CGG TTG ATK TCC TTG A	736		
ITS1 - ITS2	Bd1a	CAG TGT GCC ATA TGT CAC G	300		Annis et al. 2004
	Bd2a	CAT GGT TCA TAT CTG TCC AG	300		
	STerF	TGC TCC ATC TCC CCC TCT TCA	1513		Martel et al. 2013
	STerR	TGA ACG CAC ATT GCA CTC TAC	1513		

2.7 Sequence analysis

The raw Sanger sequencing data were visualized, trimmed and assembled using Geneious 9.1.6 (Kearse et al. 2012). Sequences were checked for chimeras using DECIPHER v.9.2 (Wright et al. 2012). Taxonomic classifications were made by contrasting the obtained 16S rRNA and *rpoD* gene sequences with representative genomes and type material from the curated public databases Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/) and the National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/), respectively. Sequences with

>97% (*rpoD*) and 99% (16S) identity were assigned to the same phylotype (Mulet et al. 2009, Edgar, 2017; Edgar, 2018). DNA sequences were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (pending accessions).

The pair-end reads from Illumina were assembled with FLASH (Magoc & Salzberg, 2011) and pre-processed using the CD-HIT software package (Li & Godzic, 2006; Fu et al. 2012). Short reads were filtered out and chimeric sequences removed. To avoid false-positive base calls, only pair-end reads with >80% ratio of bases with phred quality scores above Q30, and >90% bases above Q20 were included in the analysis. Filtered, non-chimeric reads were clustered into phylotypes with QIIME 2 (Caporaso et al. 2010), using RDP as the reference database for taxonomy assignment with a threshold value of 97% identity. To estimate bacterial community richness and diversity, the Chao1 richness estimator and the Shannon and Inversed Simpson diversity indices were used; the statistics and graphics were produced in QIIME 2. Bacterial phyla with relative abundances above 3% were regarded as dominant (Bird et al. 2018). Representative sequences of each phylotype cluster were deposited in GenBank (pending accessions).

3. Results

3.1 Study site and sampling

To study the community of resident skin bacteria in a population of *Bolitoglossa nicefori*, we performed field expeditions in four months (March, June, October and December 2017) with different precipitation levels. The study area corresponds to a secondary lower montane moist forest (*sensu* Holdridge, 1987). The annual rainfall pattern of the study site consists of two peaks of rain (Figure 2). October is the wettest month with an average rainfall of 196 mm, while December and January, the driest months, reach as low as 76 and 64 mm, respectively. Rainfall

levels for March (108 mm) and June (110 mm) are nearly identical and fall below the annual average (121 mm). The mean annual temperature of the study area is 18.2 ºC with an average minimum of 13.6 ± 0.36 ºC and an average maximum of 22.7 ± 0.35 ºC (Figure 2). In total, 42 specimens of *Bolitoglossa nicefori* were found either foraging on the leaves of ferns, herbaceous plants and the branches of young trees, or within the leaf litter and soil on the forest floor. We swabbed the whole specimen to obtain a more precise representation of the skin microbiota (Culp et al. 2007). Swab samples from March and June were used for bacterial culturing. Conversely, swabs taken in October and December were used for the metagenomic analysis of uncultured samples to contrast the bacterial community in the wettest and driest months of the year (i.e., October and December, respectively).

3.2 Bacterial culturing

To optimize the culturability of skin bacteria under laboratory conditions, we attempted to replicate the environmental conditions from the study area. As we collected the samples, we registered the relative humidity and ambient temperature, as well as the surface temperature of the skin and substrates where the salamanders were found. The average relative humidity of the sampled months was 80% (max. 89% and min. 62%) and the average ambient temperature was 18 ºC (max. 25 ºC and min. 14 ºC). The average epidermal temperature of the salamanders was 17 ºC. For every individual, the epidermal and substrate temperature were identical. Considering the environmental data, we decided to culture our samples at 20 ºC and 70-80% relative humidity. Furthermore, to maximize the number of bacterial isolates recovered by plate culture, we tried four non-selective agar media: R2A, CYC, TSA and LB. Among the culture media used in this study, R2A recovered the highest diversity of cultured bacteria. R2A (Reasoner and Geldreich, 1985) is a general purpose, low-nutrient medium suitable for the isolation of slow-growing bacteria, widely

used in amphibian microbiota studies (Lauer et al. 2007; Lauer et al. 2008; Walke et al. 2015; Medina et al. 2017). The superiority of R2A over high-nutrient media like TSA and LB for the cultivation of amphibian skin associated bacteria has been shown recently (Medina et al. 2017). CYC was optimal for visualization and distinction of bacterial morphology. While most bacterial colonies grew flat, pale or translucent on R2A, their pigmentation and morphological features were accentuated on CYC. By contrast, some isolates exhibited production of extracellular fluorescent pigments in R2A but not on CYC. For these reasons, we selected R2A as the main medium for bacterial culture and cryopreservation while CYC was used for the visualization of colony morphology. To obtain pure bacterial isolates from total cultures, we selected colonies based on their unique set of phenotypic features. As a result, we detected and isolated 32 distinct morphotypes of epibiotic bacteria from the skin of *Bolitoglossa nicefori*.

3.3 Composition and characteristics of bacterial isolates

For bacterial identification, we sequenced the 16S rRNA gene from the isolate bacteria and aligned against the RDP and NCBI databases. Our alignment resulted in sequences having 100% identity with several species of a same genus, especially in the case of *Pseudomonas* species. For this reason, we were unable to classify most of our isolates to the species level. Thus, using a 99% identity threshold we assigned each sequenced isolate to a reference genus in the database, following previous works (Edgar, 2017; Edgar, 2018). Even though the 16S rRNA gene is the universally used marker in bacterial taxonomy, the occurrence of multiple and variable intragenomic copies in a single organism along with its low taxonomic resolution at the genus or species level can lead to underestimations of the bacterial diversity (Mulet et al. 2009; Větrovský & Baldrian, 2013). Particularly within *Pseudomonas*, around 4 to 7 rrn operons have been identified (Mulet et al. 2009). A second approach to classifying our *Pseudomonas* isolates

consisted on sequencing the protein-coding *rpoD* gene. We chose this molecular marker because it is present in single copy and exhibits higher resolution than the 16S rRNA gene at the intrageneric level within *Pseudomonas*, a complex taxonomic group (Mulet et al. 2009; Mulet et al. 2010; García-Valdés & Lalucat, 2016). As a result, our 32 morphotypes were assigned to 23 phylotypes belonging to 12 genera and 9 families within the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (Table 2). The Proteobacteria (Beta- and Gamma-proteobacteria) exhibited the highest richness of cultured phylotypes and, within this phylum, *Pseudomonas* was the most widely represented genus in our samples, with eleven phylotypes.

Table 2. Composition of bacterial strains isolated from the skin of *Bolitoglossa nicefori* (GenBank accessions pending).

Phylum	**Class**	**Family**	**Isolate**	**Accessions**	
				16S	***rpoD***
Actinobacteria	Actinobacteria	Micrococcaceae	*Arthrobacter* sp.	*	
		Microbacteriaceae	*Microbacterium* sp.	*	
Bacteroidetes	Flavobacteria	Flavobacteriaceae	*Chryseobacterium indologenes*	*	
Firmicutes	Bacilli	Bacillaceae	*Bacillus* sp.	*	
			Exiguobacterium sp.	*	
Proteobacteria	Betaproteobacteria	Alcaligenaceae	*Achromobacter* sp.	*	
	Gammaproteobacteria	Enterobacteriaceae	*Citrobacter* sp.	*	
			Klebsiella sp.	*	
			Serratia sp.	*	
		Moraxellaceae	*Acinetobacter* sp. 1	*	
			Acinetobacter sp. 2	*	
		Xanthomonadaceae	*Stenotrophomonas* sp.	*	
		Pseudomonadaceae	*Pseudomonas fluorescens*	*	*
			Pseudomonas panacis	*	*
			Pseudomonas putida	*	*
			Pseudomonas tolaasii	*	*
			Pseudomonas sp. 1	*	*
			Pseudomonas sp. 2	*	*
			Pseudomonas sp. 3	*	*
			Pseudomonas sp. 4	*	*

Pseudomonas sp. 5	*	*
Pseudomonas sp. 6	*	
Pseudomonas sp. 7	*	

To aid in bacterial classification, we registered the morphological characteristics of each cultured isolate in CYC. Interestingly, we observed phenotypic plasticity among our cultured *Pseudomonas* species. Some isolates showed clear differences in pigmentation and/or colony morphology while growing under the same culture conditions (Appendix 3 A-D). Nevertheless, after analysing the *rpoD* gene sequences these isolates were assigned to a single phylotype with 100% identity to a reference species in the GenBank database. In addition, some *Pseudomonas* isolates exhibited large, pulvinate and heavily mucoid colonies that produced large quantities of extracellular matrix in plate cultures (Appendix 4 A, B), independently of the culture media. Colonies of *Achromobacter* sp. exhibited a wrinkled pattern of morphogenesis in CYC plates (Appendix 4 C, D), which is characteristic of bacteria that undergo biofilm formation (Haussler & Fuqua, 2013; Wang et al. 2016). Iridescence of bacterial colonies was observed in most of the isolates with transillumination, except for strains of *Arthrobacter* sp., *Chryseobacterium indologenes* and *P. tolaasii* (Appendix 5). In strains of *Acinetobacter sp*., iridescence was accompanied by a form of bacterial motility, probably swarming, of non-flagellated bacteria (Kaiser, 2007), or other type of motility like twitching or gliding (Kearns, 2010; Eijkelkamp et al. 2011), as evidenced by the formation of a halo growth pattern (Appendix 6 A). Iridescence was not detected with epi-illumination or exposure under natural light. By contrast, fluorescence at 365 and 395 nm was exclusively visualized in strains of *Pseudomonas* spp. (e.g., Appendix 6 B), and a strain of *Exiguobacterium* sp., by means of secretion of extracellular pigments that changed the appearance of the medium in plate cultures. Fluorescence was visualized on R2A plates but not on CYC cultures.

3.4 Metagenomic libraries derived from broth-enrichment cultures

It is known that fast-growing bacteria can mask or suppress slow-growing bacteria, particularly when grown in high-nutrient media (Walke et al. 2015). Therefore, to have a more accurate representation of the cultivable fraction of the skin-associated bacteria, we constructed a 16S rRNA gene library from the enrichment cultures using the Illumina MiSeq platform. We obtained 82,939 sequence reads, after filtering and eliminating chimeric sequences. The analysis revealed a total of 112 OTUs belonging to 24 families within the phyla Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria and Verrucomicrobia (Figure 3 A, B). The composition and proportion of OTUs varied between samples from March and June. In general terms, the diversity and richness of cultivable OTUs was higher in March (Table 3). In samples from March, the Proteobacteria (families Enterobacteriaceae, and Pseudomonadaceae) and Firmicutes (family Streptococcaceae) comprised 84% and 16% of all sequence reads, respectively (Figure 3 A; Appendix 7 A). By contrast, the phyla Bacteroidetes (families Flavobacteriaceae and Sphingobacteriaceae) and Proteobacteria (family Pseudomonadaceae) were dominant in samples from June comprising 78% and 22% of all sequence reads, respectively (Figure 3 B; Appendix 7 B). Non-dominant families comprised less than 3% of the total sequence reads. In general, next generation sequencing detected a high number of underrepresented OTUs present in the enrichment cultures that could not be isolated by standard plate culturing methods.

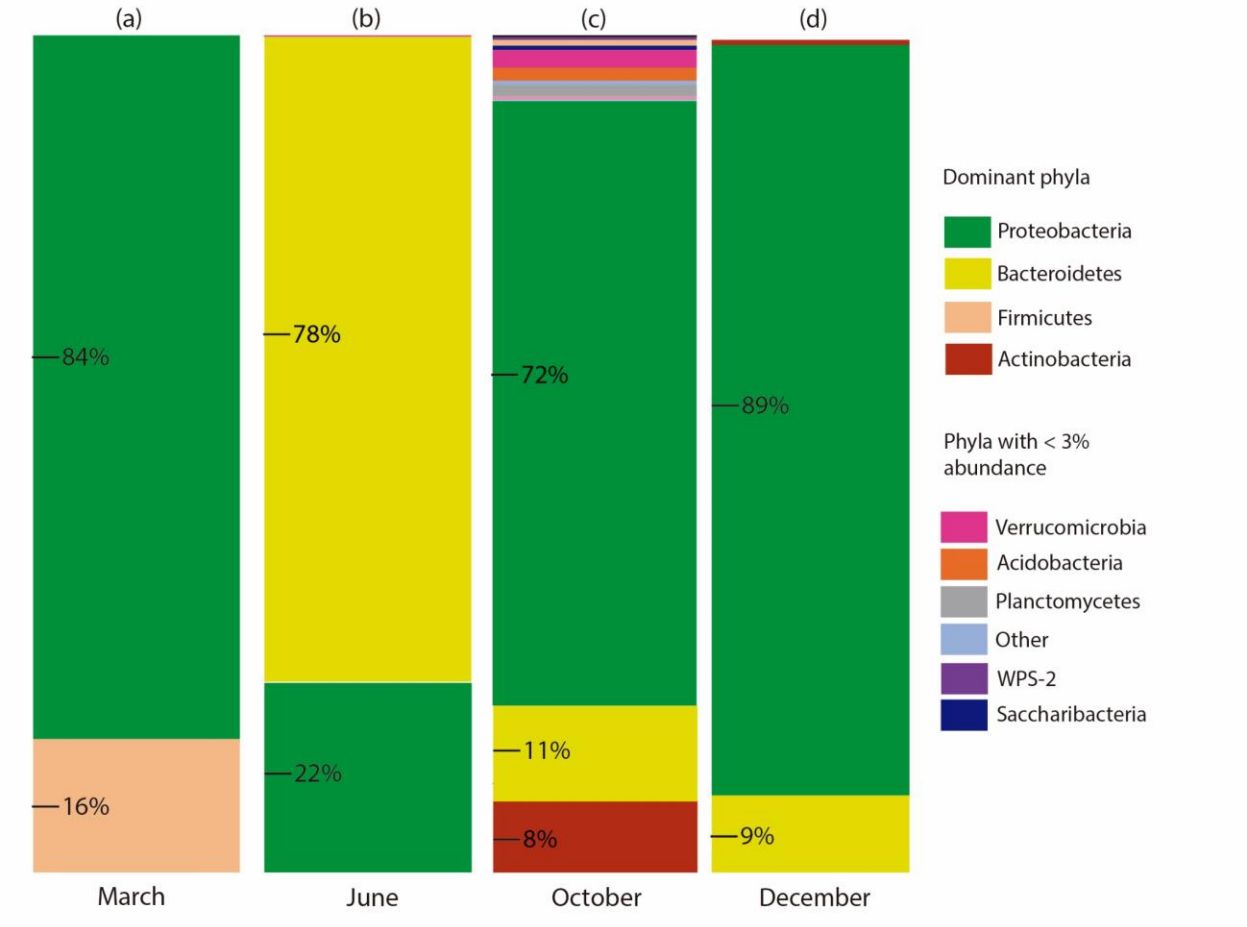

Figure 3. Composition and relative abundance of the dominant phyla that comprise the skin microbiota of *B. nicefori* in the cultured and uncultured samples from the different sampling months.

Table 3. Richness and diversity estimates for each month sampled.

Sampling month	Phylotypes	Chao1	Shannon	Simpson
March	45	45.33	2.66	0.77
June	67	88	2.23	0.61
October (wettest month)	622	623	5.86	0.87
December (driest month)	201	202	4.14	0.89

3.5 Metagenomic analysis of whole bacterial community

Another approach to uncovering the diversity of the skin microbiota in this population of *Bolitoglossa nicefori* consisted on sequencing the 16S rRNA gene with Illumina MiSeq. As a

result, we obtained 358,961 sequence reads of which 146,213 reads were included in the analysis after denoising (i.e., removal of short, chimeric or low-quality sequence reads, pseudogenes and sequences with ambiguous base calls). The microbial community of *B. nicefori* skin was represented by 823 phylotypes predominantly from the phyla Proteobacteria (82%), Bacteroidetes (10%) and Actinobacteria (4%), (Figure 3 B, C). In general, there was a high taxonomic diversity marked by low abundances, with most genera found in proportions below 1% (i.e., less than 650 reads). The most abundant families were Pseudomonadaceae (42%), Comamonadaceae (9%), Enterobacteriaceae (8%), Flavobacteriaceae (5%), Rhizobiaceae (4%) and Sphingobacteriaceae (3%). Interestingly, some sequences (<1%) were assigned to poorly known higher taxonomic groups: we detected 392 sequence reads from the phylum Saccharibacteria (formerly candidate division TM7), 250 reads assigned to candidate division WPS-2 and 467 reads that could not be classified into any known bacterial phylum. The composition and proportion of phylotypes differed between the two sampled seasons, particularly at the family level. The bacterial community from the driest month consisted of 201 phylotypes (81,047 reads) and was dominated by members of Proteobacteria (89%) and Bacteroidetes (9%) (Figure 3 B). There were 6 abundant families (Pseudomonadaceae-48%, Enterobacteriaceae-11%, Comamonadaceae-10%, Flavobacteriaceae-6%, Moraxellaceae-5% and Sphingobacteriaceae-3%) and 44 underrepresented families with less than 1% of phylotypes (Appendix 7 C). We found 622 phylotypes in samples from the wettest month, mostly from the phyla Proteobacteria (72%), Bacteroidetes (11%) and Actinobacteria (8%) (Figure 3 C). We registered 5 dominant families (Pseudomonadaceae-35%, Comamonadaceae-8%, Rhizobiaceae-6%, Enterobacteriaceae-5%, Sphingomonadaceae-3%) and 110 families in proportions below 1% (Appendix 7 D).

To compare the richness and diversity of skin-associated bacteria between the driest and wettest months, we calculated the non-parametric Chao1 richness estimate, as well as the Shannon and Simpson diversity indexes (Table 3). Chao1 performs particularly well with datasets with low-abundance groups, though, at low sample sizes, true richness can be underestimated (Hughes et al. 2001). The observed number of phylotypes and the calculated Chao1 indicate a higher richness of phylotypes in samples from the wettest month (623 phylotypes) compared to the driest month (202 phylotypes). Similarly, a greater bacterial diversity was exhibited in samples from the wettest month in contrast with the driest month, as revealed by the Shannon index. On the other hand, the Simpson index was very similar in both sampling months. This diversity index is influenced by dominant species and is little affected by underrepresented, low-abundant species. In this case, the majority of phylotypes from the wettest and driest months were represented by less than 1% of sequence reads and only a few phylotypes were dominant.

3.6 Detection of skin pathogenic fungi

Detection of the chytrid *Batrachochytrium dendrobatidis* (*Bd*) is usual in studies of the amphibian skin microbiota (e.g., Becker & Harris, 2010; Bird et al. 2018; Varela et al. 2018), given its impact on worldwide amphibian populations and the implications for their conservation (Van Rooij et al. 2015). Moreover, a newly described chytrid, *Batrachochytrium salamandrivorans* (*Bsal*), is specialised in infecting salamanders (Martel et a. 2013); this chytrid originated in south-east Asia and was recently introduced to Europe, having dramatic effects on native salamander populations (Martel et al. 2014; Spitzen-van der Sluijs et al. 2016). Even though previous studies have failed to detect *Bsal* in America (e.g., Martel et al. 2014; Parrot et al. 2017), preventive surveillance measures should be taken. In this sense, to detect the presence of the skin pathogenic fungi *Bd* and *Bsal* in this population of *B. nicefori*, we amplified the ribosomal intergenic spacers ITS1-ITS2

and the 5.8S rRNA gene with specifically designed primer sets. All captured salamanders from this population tested negative for both *Bd* and *Bsal*. In addition, no specimens were found dead or with apparent disease marks. Given that the natural skin-microbiota play a protective role in amphibians against pathogenic fungi (Banning et al. 2008; Becker & Harris, 2010), knowledge of the inhibitory potential of cultured bacteria can be helpful for the design of antifungal probiotic cocktails (Harris et al. 2009). For this reason, we aligned our sequenced isolates with published datasets of amphibian skin bacteria with known *Bd*-inhibitory status to explore the inhibitory or enhancing potential of our cultured isolates (Woodhams et al. 2015; Catenazzi et al. 2018). The analysis of the sequence data revealed that some of our isolates had more than 98% identity with both *Bd*-inhibitory and *Bd*-enhancing strains, as well as strains with a neutral effect on *Bd* growth in plate culture.

4. Discussion

The resident community of skin-associated bacteria in the mushroomtongue salamander *Bolitoglossa nicefori* was represented primarily by phylotypes within the phyla Proteobacteria, Bacteroidetes and Actinobacteria (Figure 3). Overall, there was a high diversity of low-abundance or rare bacterial phylotypes and a few dominant taxa composing the core community. Particularly, members of the genus *Pseudomonas* (Gammaproteobacteria), dominated the core community accounting for 42% of all sequence reads. This uneven abundance distribution of bacterial phylotypes is consistent with other studies showing that although the amphibian skin microbiota originates from the environment, only some taxa successfully colonize and dominate the amphibian skin (Culp et al. 2007; Loudon et al. 2014; Belden et al 2015; Sanchez et al. 2016; Bletz et al. 2017). This seems to be a generalized pattern in amphibians independent of geographic location and phylogenetic relatedness, given that several studies have found a similar composition

of the cutaneous bacterial community structure in other species of salamanders and frogs, especially in species with terrestrial habits (Lauer et al. 2007; Loudon et al. 2014; Belden et al. 2015; Longo et al. 2015; Vences et al. 2015; Walke et al. 2015; Sanchez et al. 2016; Bletz et al. 2017; Muletz-Wolz et al. 2018; Prado-Irwin et al. 2017; Bird et al. 2018; Varela et al. 2018). Species of *Pseudomonas* are widespread in the environment; they are naturally found in soil and water or as pathogens of plants and animals (García-Valdés & Lalucat, 2016). Some species of fluorescent *Pseudomonas*, such as those found in the present study, synthesize phenazines, which exhibit antibiotic activity against bacteria, fungi, plant and animal tissues (Mavrodi et al. 2006; Pierson & Pierson, 2010). The production of strong broad-spectrum secondary metabolites like phenazines might explain in part why *Pseudomonas* are that competitive and successful at colonizing and dominating the skin microbial community in *B. nicefori* and amphibians in general. In amphibians, *Pseudomonas* and other anti-*Bd* skin bacteria serve an important functional role, providing host protection against fungal pathogens. In turn, symbiotic bacteria benefit from nutrients and space from the host skin (Culp et al. 2007; Lauer et al. 2007; Harris et al. 2009; Becker & Harris, 2010; Becker et al. 2015). The amphibian host could also be regarded as a means of dispersal for transient and resident bacteria as it moves throughout different substrates within the microhabitat. This symbiotic relationship between skin bacteria and the amphibian host resembles what has been described as an asymmetric dependence (Minter et al. 2018). Being environmentally derived, skin bacteria can likely survive without its host. On the other hand, despite the host innate defences, amphibians rely on skin symbionts to combat colonization of invading pathogens and perform other important functions (Loudon et al. 2014). For instance, a new study showed that skin secretions of some salamanders can effectively kill both *Bd* and *Bsal* zoospores, while other species are highly susceptible to these chytrids (Smith et al. 2018).

We found differences in the composition and relative abundance of bacterial phylotypes between the wettest and driest months of the year (i.e., October and December, respectively). Samples from the wettest month had a considerably higher diversity mostly represented by low abundance phylotypes. However, the bacterial community structure of both sets of samples shared the same dominant phyla, except for the Actinobacteria, which was reduced from 8% of relative abundance to less than 1% in samples from the driest month (Figure 3 C, D). Because amphibians obtain and select bacteria from their environment, it is reasonable to infer that the seasonal changes observed in the diversity of skin bacteria could be partly attributed to the dynamics of the soil microbial community in response to fluctuating precipitation levels. This is considering that other environmental factors like ambient temperature and relative humidity remained relatively stable during the sampling months. Seasonal variability in soil moisture (i.e., desiccation and rewetting) is usually accompanied by changes in pH, electrical conductivity and nutrient availability (e.g., phosphorus, nitrogen, carbon), which are the main drivers of soil bacterial diversity and abundance along with plant productivity (Shilpkar et al. 2010; Cregger et al. 2012; Koorem et al. 2014; Meisner et al. 2015; Zhou et al. 2016; Delgado-Baquerizo et al. 2018). It has been shown however that phylotypes that are dominant in the amphibian skin are usually found at low abundance levels in their surrounding environment (Walke et al. 2014; Bates et al. 2018), particularly because of host filters (Longo et al. 2015; Muletz-Wolz et al. 2018).

Our findings suggest that the core community of skin bacteria in this population of *B. nicefori* is resilient to environmental perturbation like seasonal variation in moisture levels, meaning that most of their functional diversity might remain stable both during the rainy and dry seasons of the year. Similarly, a new study tested the effects of soil pH and precipitation levels on the

cutaneous microbial diversity in three species of frogs and found some differences in the abundance of bacterial phylotypes from before and after the onset of the wet season, but the observed bacterial richness was not significantly affected (Varela et al. 2018). Another study examined the influence of seasonal transition from summer to winter on the stability of the composition and structure of the skin microbial community in a population of the lowland leopard frog *Lithobates yavapaiensis*. Significantly higher bacterial richness was observed in specimens sampled during the winter season, when the species immune system is most susceptible to skin microbial infections (Longo et al. 2015). In this study we observed that elevated rainfall levels favoured colonization or increased abundance of new and rare bacterial phylotypes belonging to candidate division WPS-2 (Nogales et al. 2001), candidate division Saccharibacteria (Ferrari et al. 2014) and the recently described phylum Armatimonadetes (Lee et al. 2013). Interestingly, a small proportion of these new phylotypes could not be assigned to any currently known phyla. Other groups that were only detected in samples from the wettest month include the phyla Acidobacteria, Planctomycetes and Verrucomicrobia, which are common soil inhabitants (Delgado-Baquerizo et al. 2018).

We detected a total of 112 OTUs in mixed enrichment cultures from skin swab samples of *Bolitoglossa nicefori* using Illumina MiSeq. By contrast, we were only able to detect and isolate 32 morphotypes belonging to 23 phylotypes based on morphological characteristics observed in plate cultures. A similar number of skin isolates has been cultured from a variety of amphibian hosts (Woodhams et al. 2015). It is likely that underrepresented or slow-growing bacteria might have been rapidly outgrown or even suppressed by competing, dominant and fast-growing species, as suggested by studies on bacterial competition in mixed cultures (Hibbing et al. 2010), especially in plate cultures obtained from serial dilutions of the initial

inoculum. It is also possible that the composition of the culture media or the culture conditions were not optimal for the formation of colonies of bacteria with specific nutritional requirements (Stewart, 2012). This could be the case of members of the phylum Verrucomicrobia, which inhabit strictly anoxic environments (Spring et al. 2016) and were present in our cultured samples with less than 1% relative abundance. Another possibility is that the chemical components of mucous secreted by salamanders during swabbing could have tampered bacterial growth, except for species that may be resistant to these molecules, probably members of the dominant phyla. Consequently, the identification of bacterial strains by visual inspection in plate cultures may underestimate the actual diversity of culturable bacteria in amphibian skin samples. Despite this, our isolates were representative of the most dominant phyla within the skin microbial community, consistent with previous studies (Walke et al. 2015). Most of the phylotypes that could not be isolated on plate cultures consisted of members of the families Comamonadaceae, Flavobacteriaceae, Sphingobacteriaceae, Enterobacteriaceae, Streptococcaceae, Aeromonadaceae, Leuconostocaceae, Enterococcaceae and Paenibacillaceae. We estimate that the culturable fraction of the skin microbiota in our study species corresponded to approximately 13% of the total bacterial richness if we count the cultured phylotypes identified with next generation sequencing, compared to 3% if we only consider isolates obtained by plate culture. The latter estimate resembles the calculated fraction of culturable skin bacteria in other amphibian microbiota studies (Walke et al. 2015). Most of the cultured isolates comprised gram-negative bacteria, consistent with a previous work with the plethodontid salamander *Plethodon cinereus* (Culp et al. 2007). In accordance with our findings from the culture-independent approach, the genus *Pseudomonas* was overrepresented

in our samples showing the highest number and abundance of culturable phylotypes, particularly strains of the *Pseudomonas fluorescens* species complex.

Among the most interesting features of our cultured *Pseudomonas* isolates we highlight the existence of phenotypic plasticity and the overproduction of exopolysaccharides (EPS), resulting in highly mucoid colonies. We ignore the factors behind the observed phenotypic plasticity; however, the production of fluorescent pigments such as phenazines are known to affect colony morphology in *Pseudomonas* (Dietrich et al. 2008). An important constituent of the EPS polymers secreted by pseudomonads is alginate, which is known to influence biofilm development and architecture (Chang et al. 2007). Production of alginate is stimulated by environmental conditions like low water availability. Given its hygroscopic properties, alginate serves a double role by inducing biofilm formation and protecting colonies from desiccation (Chang et al. 2007). In amphibians, the production of EPS and biofilm development by skin bacteria could facilitate colonization and create a protective layer against invading pathogens and dehydration (Chang et al. 2007; Ma et al. 2012). Mucoid bacterial colonies have been isolated from skin samples in other plethodontid salamanders like *Plethodon cinereus* (Culp et al. 2007). Finally, we observed iridescence, a form of structural colour, in almost all our cultured isolates. Although iridescence has been reported in *Pseudomonas* and other bacterial taxa (mostly marine bacteria like *Cellulophaga lytica*), it is rarely documented in the literature and its significance or the mechanisms that govern this phenomenon are still poorly understood (Kientz et al. 2012; Kientz et al. 2016, Chapelais-Baron et al. 2018). A new study by Chapelais-Baron et al., (2018) showed a link between bacterial iridescence and the presence of gliding motility. The authors hypothesize about the photoprotective potential of iridescent molecules,

which could be beneficial to the amphibian host given their susceptibility to UV radiation (Alton & Franklin, 2017).

The salamanders sampled in this population of *B. nicefori* showed no apparent signs of disease and tested negative for the pathogenic fungi *Batrachochytrium dendrobatidis* and *B. salamandrivorans*. *Bd* is known to disrupt the natural microbial community structure in infected amphibian populations (Jani & Briggs, 2014), possibly leading to misrepresentations of the normal host microbiota of healthy individuals. Our results suggest that the studied population of *B. nicefori* might be free of the fungi that cause chytridiomycosis or if the pathogen is indeed present, it might be found at undetectable levels or may show a low prevalence. Comparisons of our cultured isolates with public databases of amphibian skin bacteria (Woodhams et al. 2015; Catenazzi et al. 2018) supported the idea that the 16S rRNA gene is not a good predictor of bacterial inhibitory potential; instead, co-culture assays provide a more reliable measure of *Bd* inhibition capacity and strength by exposing a host bacterial strain to the fungal pathogen (Catenazzi et al. 2018). Even though we did not perform challenge assays, we observed high alpha diversity of skin bacterial phylotypes, which can be a better indicative of the protective potential of the whole bacterial community, against invading pathogens (Piovia-Scott et al. 2017; Bates et al. 2018; Catenazzi et al. 2018). For instance, members of the Bacteroidetes and Proteobacteria, especially *Pseudomonas*, are known for being composed predominantly of *Bd*-inhibiting isolates (Lauer et al. 2008; Becker et al. 2015). However, given that anti-*Bd* isolates cannot inhibit a broad spectrum of *Bd* genotypes (Bletz et al. 2017b), co-culture assays are still important to evaluate host susceptibility against specific *Bd* genotypes. In this sense, *in vitro* cultures of amphibian skin cell lines might serve as a study model to better understand the additive or synergistic interactions between probiotic bacterial symbionts in the amphibian skin

and their inhibitory potential against fungal pathogens (Loudon et al. 2014b), resembling similar studies of the human skin microbiota (Bojar, 2015). The bacterial isolates obtained in this study could be eventually challenged with a set of *Bd* genotypes to determine their *Bd* inhibitory status, which could be helpful in the event of infection with chytridiomycosis in this studied population.

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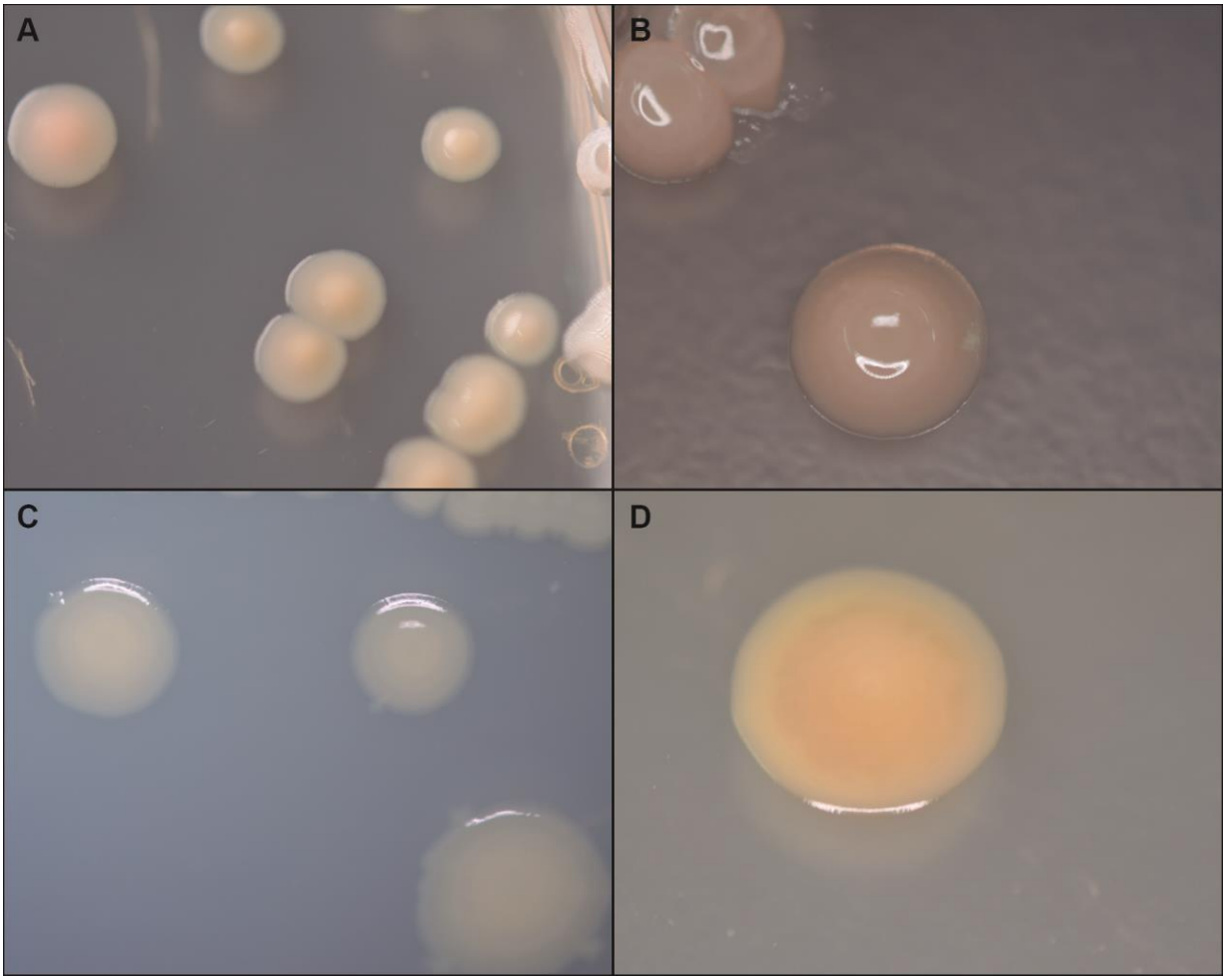
Appendix

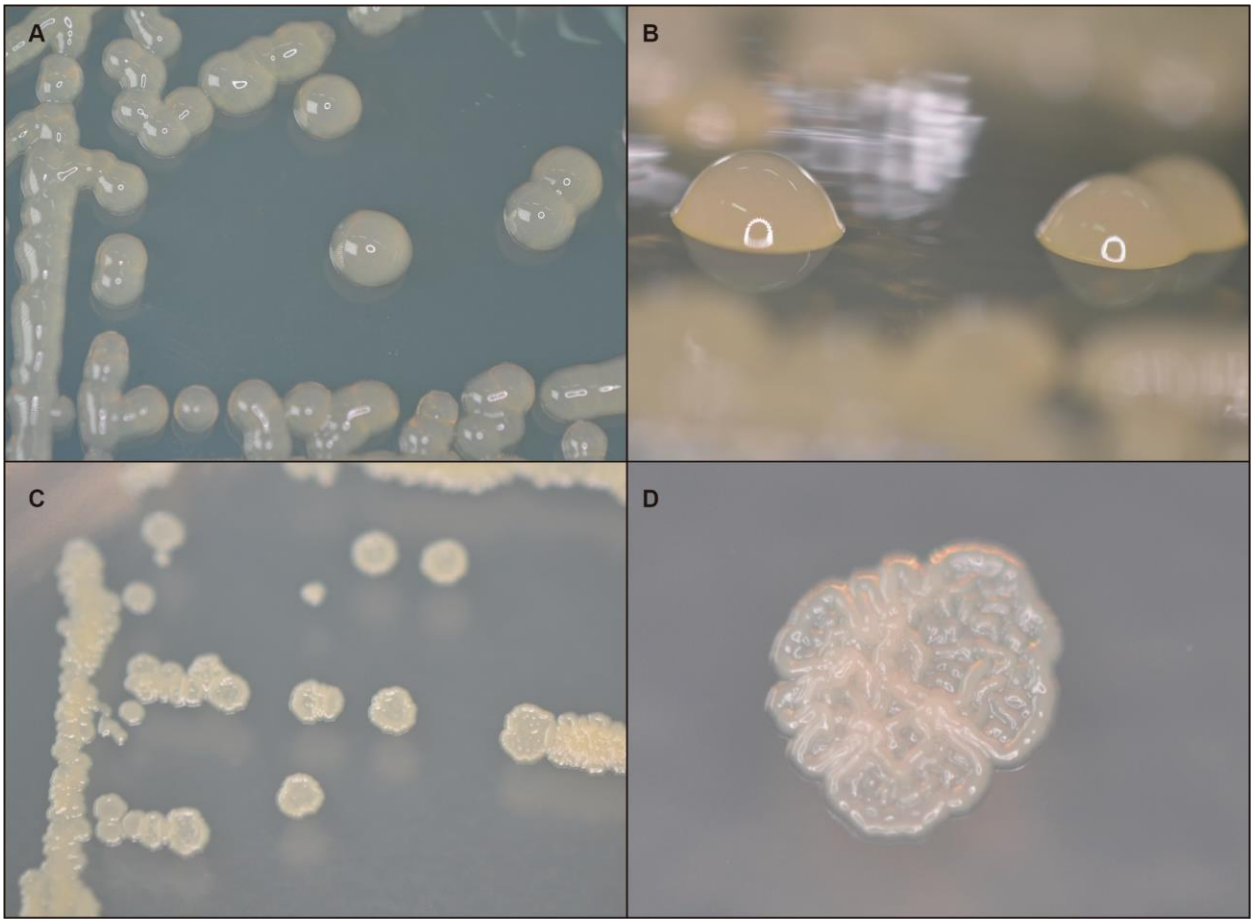
Appendix A. Composition of culture media used for the cultivation of skin-associated bacteria isolated from Bolitoglossa nicefori.

Component (g/L)	Medium			
	R2A	**CYC**	**TSA**	**LB**
Casamino acids	-	6.1	-	-
Casein acid hydrolysate	0.5	-	-	-
Dextrose	0.5	-	-	-
Enzymatic digest of soya bean	-	-	5	-
$FeSO_4.7H_2O$	-	0.01	-	-
K_2HPO_4	0.3	-	-	-
K_2HPO_4	-	1	-	-
KCl	-	0.5	-	-
$MgSO_4$	0.024	-	-	-
$MgSO_4.7H_2O$	-	0.5	-	-
NaCl	-	-	5	10
$NaNO_3$	-	3	-	-
Pancreatic digest of casein	-	-	15	-
Proteose peptone	0.5	-	-	-
Sodium pyruvate	0.3	-	-	-
Starch	0.5	-	-	-
Sucrose	-	30	-	-
Tryptone	-	-	-	10
Yeast extract	0.5	2	-	5
Agar	15	15	15	15
pH	7.2 ± 0.2	7.2 ± 0.2	7.3 ± 0.2	7.5 ± 0.2

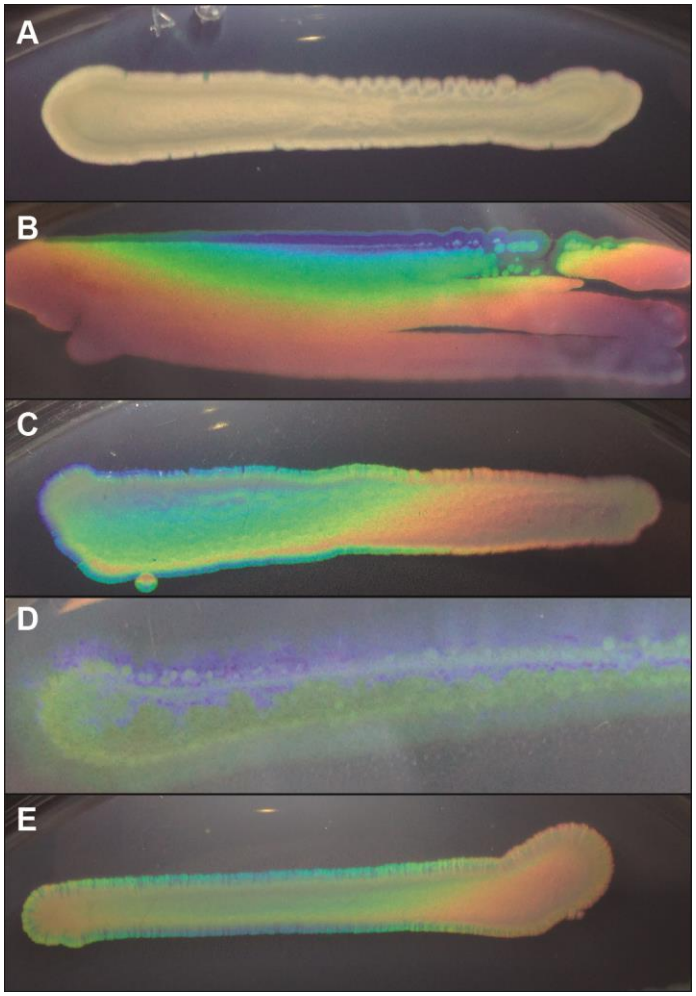
Appendix B. Photographs of the study site (A), study species, Bolitoglossa nicefori (B) and sampling method (C).



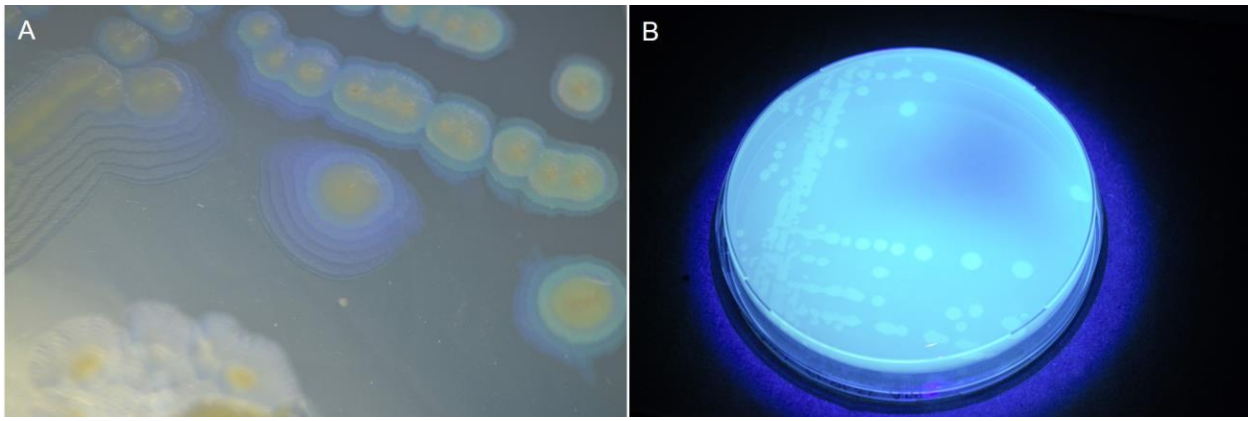
Appendix C. Phenotypic plasticity observed in Pseudomonas isolates showing different morphotypes that belong to a same phylotype.



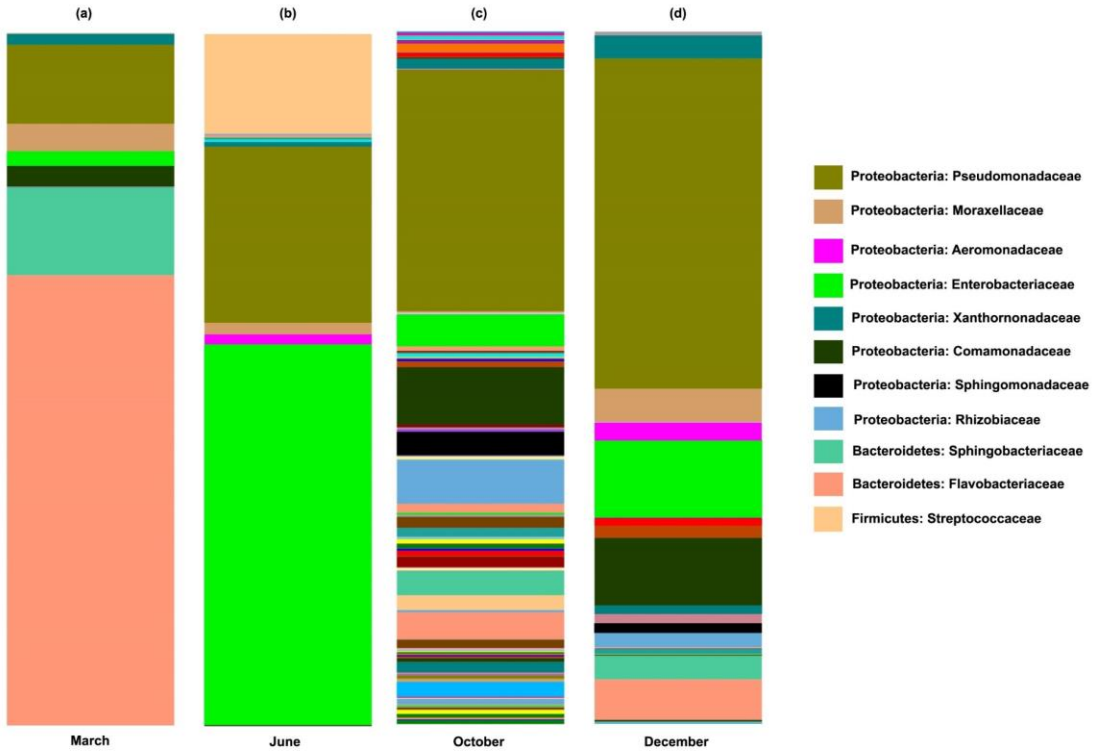
Appendix D. Morphological characteristics of some cultured isolates. A-B, mucoid colonies of Pseudomonas sp. C-D, Colonies of Arthrobacter sp. showing a wrinkled pattern of morphogenesis.



Appendix E. Iridescence in some bacterial isolates. A, non-iridescent isolate of *Arthrobacter* sp. B, *Acinetobacter* sp. C, *Pseudomonas* sp. D, *Acinetobacter* sp2. E, *Serratia* sp.



Appendix F. Characteristics of some bacterial isolates. A, bacterial motility in Acinetobacter sp., showing the formation of a halo growth pattern. B, Pseudomonas fluorescens exposed to UV light.



Appendix G. Composition and abundance of culturable and unculturable bacterial families in the different sampling months.