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Genetic analysis of post-epizootic amphibian chytrid strains in Bolivia: Adding a piece to the puzzle

Patricia A. Burrowes[1] | **Timothy Y. James**[2] | **Thomas S. Jenkinson**[3] | **Ignacio De la Riva**[4]

[1]Department of Biology, University of Puerto Rico, San Juan, Puerto Rico

[2]Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI, USA

[3]Department of Environmental Science, Policy, and Management, University of California, Berkeley, CA, USA

[4]Museo Nacional de Ciencias Naturales, CSIC, Madrid, Spain

Correspondence
Patricia Burrowes, University of Puerto Rico, Biology, P.O. Box 23360, San Juan 00931, Puerto Rico.
Email: pburrowesupr@gmail.com

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Abstract

The evolutionary history and dispersal pattern of *Batrachochytrium dendrobatidis (Bd)*, an emergent fungal pathogen responsible for the decline and extinctions of many species of amphibians worldwide, is still not well understood. In South America, the tropical Andes are known as an important site for amphibian diversification, but also for being a place where hosts are at greater risk of chytridiomycosis. In an attempt to understand the history and the geographic pattern of *Bd*-associated amphibian declines in Bolivia, we isolated *Bd* from hosts at two locations that differ in their chronology of *Bd* prevalence and host survival outcome, the cloud forests of the Amazonian slopes of the Andes and Lake Titicaca in the altiplano. We genotyped *Bd* from both locations and sequenced the genome from the cloud forest isolate and then compared them to reference sequences of other *Bd* strains across the world. We found that the Bolivian chytrid isolates were nearly genotypically identical and that they belong to the global panzootic lineage (*Bd*-GPL). The Bolivian *Bd* strain grouped with other tropical New World strains but was closest to those from the Brazilian Atlantic Forest. Our results extend the presence of *Bd*-GPL to the central Andes in South America and report this hypervirulent strain at Lago Titicaca, where *Bd* has been detected since 1863, without evidence of amphibian declines. These findings suggest a more complex evolutionary history for this pathogen in Bolivia and may point to the existence of an old lineage of *Bd* that has since been extirpated following the arrival of the panzootic *Bd-GPL* or that the timing of *Bd*-GPL emergence is earlier than generally acknowledged.

KEYWORDS

amphibian declines, *Batrachochytrium dendrobatidis*, chytridiomycosis, emergent infectious diseases, fungal disease, genotyping

1 | INTRODUCTION

Global amphibian declines in the Anthropocene have been associated with different factors that include land use change, contaminants, introduced species, climate change and emerging infectious diseases (see, e.g. Agostini & Burrowes, 2015; Collins & Crump, 2009; Cunningham, Hyatt, Russell, & Bennett, 2007; Daszak, Cunningham, & Hyatt, 2003; La Marca et al., 2005; Ron et al., 2003; Stuart et al., 2004). The role of two fungal pathogens, namely *Batrachochytrium dendrobatidis* (Longcore, Pessier, & Nichols, 1999, =*Bd* hereafter) and *B. salamandrivorans* (Martel et al., 2013), in the loss of amphibians has been of special concern (Fisher, Garner, & Walker, 2009; James et al., 2015; Skerratt et al., 2007; Stegen et al., 2017), and *Bd* has been associated with the decline of 501 species worldwide, including the extinction of 90 species (Scheele et al., 2019). Research points to tropical areas of South America as

one of the most affected regions and to global trade as an important facilitator of the spread of these chytrid pathogens around the world (Scheele et al., 2019).

Efforts to understand the evolution of one of these invasive chytrids (*Bd*) have revealed the genetic differences between strains and how genotypes are associated with geographical regions and virulence (Bataille et al., 2013; Farrer et al., 2011; Goka et al., 2009; O'hanlon et al., 2018; Rodriguez, Becker, Pupin, Haddad, & Zamudio, 2014; Rosenblum et al., 2013; Schloegel et al., 2012). Using whole genome sequencing of world-wide *Bd* strains, O´Hanlon et al. (2018) suggested an Asian origin of the species, because of the presence of a hyperdiverse lineage, *Bd*-ASIA-1, that bears the hallmarks of frequent sexual reproduction and the absence of disease-associated declines in the region. The lineage associated with the modern catastrophic declines reported by Scheele et al. (2019) is known as *Bd*-GPL, the global panzootic lineage (O'hanlon et al., 2018). The genomic data suggest a twentieth-century origin of *Bd*-GPL and a dispersal history that was facilitated by the global trade of amphibians; however, the 95% confidence interval on the origin includes the late nineteenth century (O'hanlon et al., 2018). Other genetically distinct strains include *Bd*-ASIA-2/*Bd*-BRAZIL which is less virulent and linked to the commercial trade of bullfrogs between Asia, Brazil and North America (Greenspan et al., 2018); *Bd*-CAPE, a less virulent strain from Africa more closely related to *Bd*-GPL; and a less virulent European strain, *Bd*-CH, related to *Bd*-ASIA-1 (O'hanlon et al., 2018). Within the *Bd*-GPL lineage, there is little evidence of population structure, making the geographic history of spread highly uncertain (Farrer et al., 2011; O'Hanlon et al., 2018; Rosenblum et al., 2013; Valenzuela-Sánchez et al., 2018). Moreover, the spread of genotypes across geographic regions has led to the emergence of hybrid genotypes which may drive rapid evolution and future panzootics of *Bd* (Greenspan et al., 2018; O'hanlon et al., 2018; Schloegel et al., 2012). Within South America, *Bd*-GPL and *Bd*-ASIA-2/*Bd*-BRAZIL lineages are present (James et al., 2015; Jenkinson et al., 2016; O'hanlon et al., 2018; Rodriguez et al., 2014; Valenzuela-Sánchez et al., 2018). Declines of Andean and Brazilian Atlantic Forest amphibians are known, and explicit hypotheses exist on the geographic direction and timing of epizootic *Bd* waves (Burrowes & De la Riva, 2017a; Lips et al., 2008); however, testing the hypotheses is hindered by the lack of historical genetic information and the paucity of cultured *Bd* strains from countries other than Brazil. There is considerably more information on *Bd* distribution than *Bd* genotypes; however, where population declines occur and *Bd* has been genotyped, it has been shown that the pathogen is of the *Bd-GPL* lineage. Few places are known to contain multiple lineages (Byrne et al., 2019; O'hanlon et al., 2018; Schloegel et al., 2012), and this may speak to the ability of *Bd-GPL* to outcompete enzootic lineages. Recent findings have shown that when *Bd-GPL* and the older Brazilian strain (now considered *Bd*-ASIA-2/*Bd*-BRAZIL; see O'hanlon et al., 2018) are co-inoculated onto a single host, *Bd-GPL* grows much faster, suggesting that it has the potential to outcompete endemic strains (Jenkinson et al., 2018). If this is the case in the wild, the global invasion of the hypervirulent *Bd-GPL* may hinder our possibility to detect ancient *Bd* strains and, thus, truly understand the evolutionary dynamics and spatial epidemiology of this pathogen. However, detection of *Bd-GPL* in the field may indicate a system in a post-epizootic state, which is a critical piece of information with respect to containment measures and conservation.

The aim of this study was to determine the *Bd* genotypes found among extant species of amphibian hosts in Bolivia, an Andean country where a once mega-diverse amphibian fauna started to decline drastically in the mid-1990s (Cortez, 2009; De la Riva, 2005; De la Riva & Burrowes, 2011; De la Riva, Köhler, Lötters, & Reichle, 2000; De la Riva & Lavilla, 2008; De la Riva & Reichle, 2014). A comprehensive study of the presence of *Bd* in the Bolivian Andean region (Burrowes & De la Riva, 2017a) revealed that *Bd:* (a) was present as early as 1863 in *Telmatobius culeus* from Lago Titicaca (the oldest record of *Bd* in the world hitherto); (b) occurred in all ecoregions from the high altiplano to inter-Andean valleys and cloud forests; (c) affected a broad taxonomic range of hosts; and (d) increased in prevalence since the mid 1990s, coincident with the timing of amphibian declines in the country. The historic and geographic pattern of occurrence of *Bd* in Bolivia suggested the presence of two *Bd* lineages: potentially, an old endemic in the high Andes where declines have not been drastic, and another, more recently introduced pathogenic lineage in the cloud forests of the Amazonian slopes of the Andes. The latter is presumed to have been responsible for the disappearance of nearly 90% of the forest *Telmatobius* and of other amphibian species in the families Hylidae, Craugastoridae and Bufonidae during the 1990s (Burrowes & De la Riva, 2017a).

In order to test this hypothesis, herein we report results on the genetics of *Bd* strains from two extant species of amphibians in this region, the hylid treefrog *Boana balzani* and the giant Titicaca water frog, *Telmatobius culeus*. We compare Bolivian *Bd* genotypes from two locations, report the first *Bd* genome sequence from the central Andes and present its relationship to other strains from a global panel. This work contributes another piece of the puzzle to a growing understanding of the spread of *Bd* and its genetic diversity (e.g. Kaiser & Pollinger, 2012; Miller et al., 2018; O'hanlon et al., 2018; Rodriguez et al., 2014; Rosenblum et al., 2013; Schloegel et al., 2012; Valenzuela-Sánchez et al., 2018).

2 | MATERIALS AND METHODS

2.1 | Strains

We followed *Bd* isolation methods proposed by Longcore et al. (1999) with slight modifications (Longcore, 2000). We used a hand lens or light microscope to screen larvae and adults for signs of chytridiomycosis using oral tissue dekeratinization (Fellers, Green, & Longcore, 2001; Fisher et al., 2018; Knapp & Morgan, 2006; Vieira et al., 2013) or thalli in excised skin pieces (toe webbing of adults). We dissected infected tissues for pathogen isolation on 1% tryptone agar with 0.2 mg/ml penicillin-G and 0.4 mg/ml streptomycin sulphate (Longcore, 2000). The cleaned pieces were then placed in new

plates that were incubated at room temperature (20–23°C), checked daily for *Bd* growth and cleaned when contaminants appeared until sufficient growth had occurred for DNA extraction. We isolated three pure *Bd* cultures from five and six tadpoles, respectively, of *Boana balzani* collected in a stream reachable from the 'Death Road' (='Carretera de la Muerte') in the yungas (=cloud forests) of Nor Yungas Province, department of La Paz, Bolivia (16°13′25″S, 67°45′16″W, at 1,440 m), during the years of 2016 (UM721) and 2017 (UM802, UM804). These samples were collected from areas (Figure 1a,b), where other species of amphibians have declined drastically since the mid-1990s (De la Riva & Reichle, 2014). An additional tissue culture from this species was attempted from the same locality in 2016 (Hb-2), and while we were unable to establish a pure culture, we were able to generate genotypes for several loci from this infected frog tissue. We sampled *Telmatobius culeus* at Lake Titicaca in Isla de la Luna, Manco Kapak Province, department of La Paz, Bolivia (16°02′42.17″S, 69°04′08″W, at 3,819 m; Figure 1c). We failed at culturing *Bd* isolated from five of the moribund adult frogs found along the shores due to contamination, but we were able to genotype *Bd* from DNA extracted from a toe-webbing sample of one of these frogs preserved in ethanol at the time (Figure 1d). Figure 2 shows the location of the two sites sampled for this study.

2.2 | DNA Methods

DNA was extracted from fungal cultures and preserved infected material (*T. culeus* ethanol preserved skin) using a 2X CTAB miniprep method (James et al., 2008). Six multilocus sequencing markers were genotyped: *Bd*C24, *Bd*C5, *Bd*SC8.10, *Bd*SC3.9, *Bd*SC3.1 and *Bd*SC7.6, using methods outlined by Jenkinson et al. (2016). Raw Sanger sequences were edited using Sequencher 5.3 (Gene Codes). Sequences were compared with existing reference data to determine whether any new haplotypes had been recovered (James et al., 2009; Jenkinson et al., 2016).

DNA from isolate UM721 and seven previously collected isolates from Brazil (CLFT043, CLFT060, CLFT085, CLFT088, CLFT100, CLFT111, CLFT131; Jenkinson et al., 2016) were used for genome sequencing (Table S1). We quantified DNA concentration in the sample using the Qubit 2.0 Fluorometer with the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Inc). We prepared short-insert (~450 bp) DNA fragment libraries according to the Nextera XT (Illumina) manufacturer's recommendations with slight modifications. Briefly, we inputted 1 ng of quantified template DNA (diluted to 0.2 ng/μl) from each sample and carried out the enzymatic fragmentation step at 55°C for 5 min before neutralizing the reaction. Then, we carried out a limited-cycle PCR to amplify and index the fragmented DNA. We dual-indexed individual samples for paired-end sequencing using the Nextera XT v2 Index Kit. Post-PCR, we purified the library by ligating indexed fragments to AMPure XP magnetic beads (Beckam Coulter Inc), and washing away impurities while retaining the beads with an Agencourt 96-well ring magnet plate (Beckam Coulter Inc). We quality-checked the fragment library for appropriate size and concentration with an Agilent 2100 Bioanalyzer (Agilent Technologies Inc). After quality control, the library was paired-end sequenced on the Illumina HiSeq 2500 platform by the University of Michigan Core DNA Sequencing Laboratory.

We assessed read quality metrics for the sample using FastQC (Andrews, 2010). We trimmed sequencing adapters and low-quality bases from the reads with Trimmomatic (Bolger, Lohse, &

FIGURE 1 Localities where animals were sampled for *Batrachochytrium dendrobatidis* isolation. Death Road ('Carretera de la Muerte') in the Bolivian cloud forests of the Amazonian slopes of the Andes (a); stream habitat of *Boana balzani* in the cloud forest (b); shores of Lake Titicaca in Isla de la Luna, where *Telmatobius culeus* were sampled (c); moribund *T. culeus* in the shores of Isla de la Luna (d)

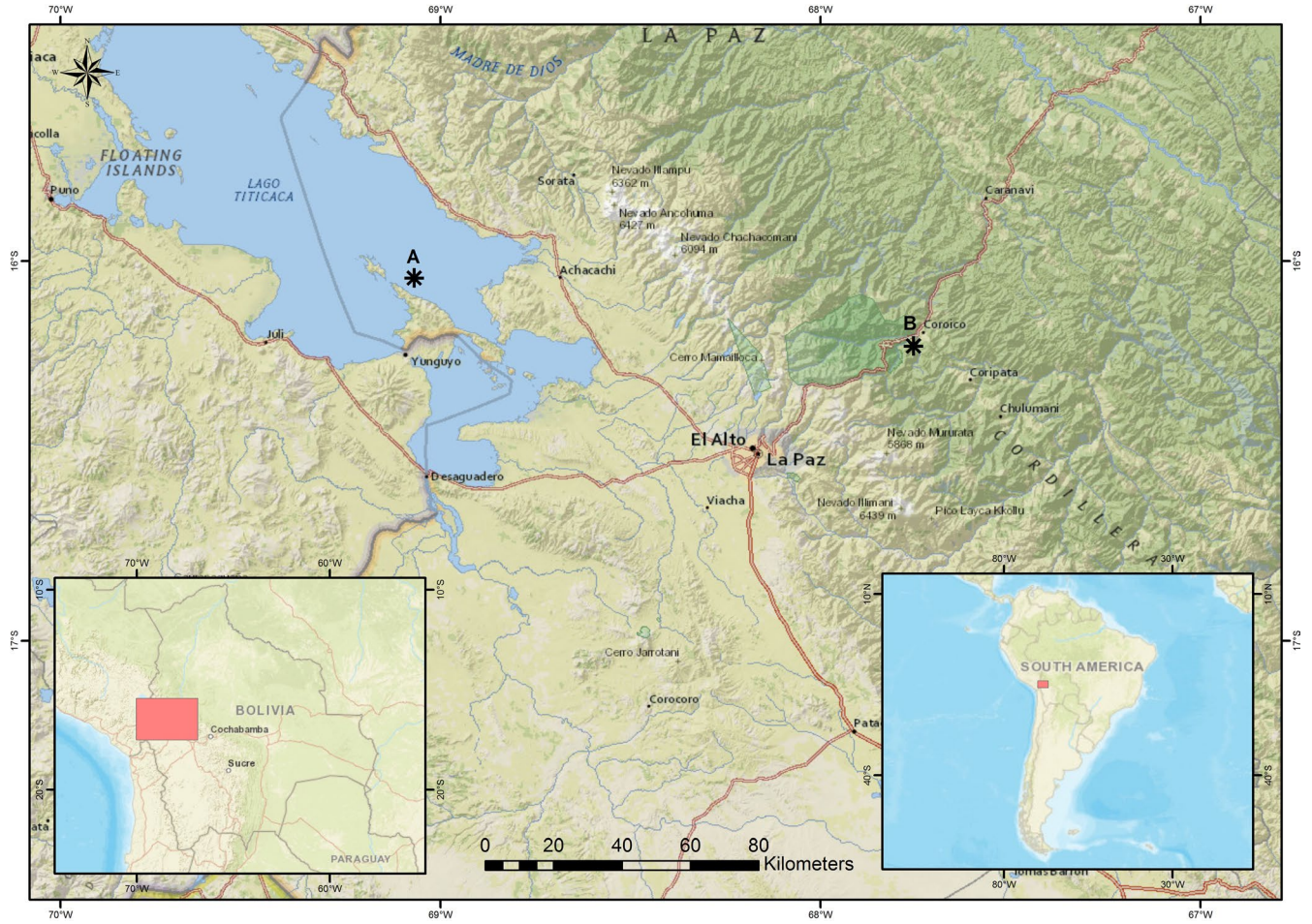

FIGURE 2 Map of the Bolivian Andes showing the two sites from where we obtained *Batrachochytrium dendrobatidis* isolates from infected frogs. (a) Isla de la Luna, within Lake Titicaca; (b) stream site along the 'Death Road' ('Carretera de la Muerte') in cloud forests (yungas)

Usadel, 2014). We assembled our reads to the *Bd* reference genome generated from strain JEL 423 (Broad Institute, version Jan. 2007) with BWA-MEM (Li, 2013). To assess the placement of our Bolivian sample within the global context, we downloaded a global panel of 49 previously published genomes (Farrer et al., 2013; O'Hanlon et al., 2018; Rosenblum et al., 2013; Valenzuela-Sánchez et al., 2018; Table S2). These downloaded reads were aligned to the JEL423 nuclear reference genome as described above. After assembly to reference, we sorted and removed duplicate reads with Picard (Broad Institute). We realigned indels, recalibrated read quality scores and indexed reads with the Genome Analysis Toolkit suite of tools (GATK; McKenna et al., 2010). We identified SNP and indel variants with GATK HaplotypeCaller and performed the final joint genotyping with GATK GenotypeGVCFs. Finally, we selected and quality-filtered SNPs to produce a final, high-confidence panel of 87,446 SNPs with GATK VariantFiltration.

We used custom perl scripts to determine genomic heterozygosity (Ho), and local average heterozygosity across a 50-kb sliding window advancing every 10 kb for each isolate. We again used custom perl scripts to determine genetic distances among our panel of isolates under a hetequal character transition matrix (Mountain & Cavalli-Sforza, 1997). We visualized the calculated distances as a neighbour-joining dendrogram using PHYLIP (Felsenstein, 1993). We evaluated statistical support for our *Bd* tree by resampling 100 bootstrap pseudo-replicates from our SNP data for distance analysis with a custom perl script.

3 | RESULTS

3.1 | Multilocus sequence typing reveals a single *Bd* lineage in the extant samples

We generated genotypes of five samples (four from *Boana balzani* [cloud forests] and one from *Telmatobius culeus* [altiplano]) using six sequence typing markers. All of the samples were identical except for UM721 and Hb-2 which were both homozygous at locus *Bd*SC3.1, while the other samples were heterozygous. The multilocus genotype of the *Bd* on *T. culeus* matched *Bd* genotypes from the cloud forest, suggesting they are part of the same lineage of closely related genotypes. Comparison of the genotypes to reference sequences (James et al., 2009; Jenkinson et al., 2016) showed that

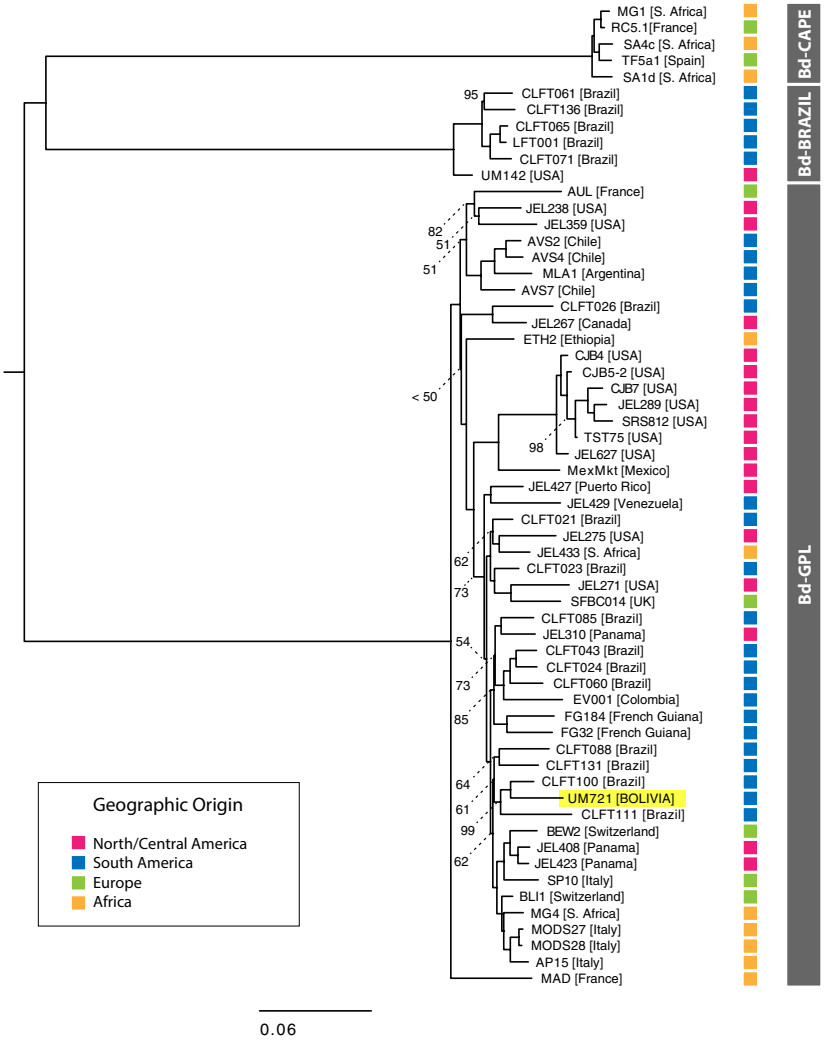

FIGURE 3 Neighbour-joining tree for *Batrachochytrium dendrobatidis (Bd)* isolates including previously published and newly sequenced isolates for this study. The tree is midpoint-rooted and based on genetic distance between 87,466 genomic SNPs. All branches are supported by 100% bootstrap support unless otherwise indicated. The major *Bd* lineages are indicated by grey bars to the right, and the locality of origin is indicated by coloured boxes

the Bolivian genotypes were similar to other members of the Global Panzootic Lineage, and like most samples from the Neotropics, lacked alleles diagnostic of the *Bd*GPL-1 lineage, characteristic of the northern temperate regions (James et al., 2015).

3.2 | Genome sequencing places Bolivian genotype with South American *Bd*-GPL strains

We sequenced the genome of the Bolivian cloud forest *Bd* strain UM721 to 363.3 X coverage (69.75 million reads) and the recently collected Brazilian strains to an average coverage of 60.2 X (range: 44.8 X–78.7 X coverage; Table S1). The data were combined with 53 published genomes to produce a dendrogram revealing the relationships of the Bolivian lineage to a global panel. The results showed that UM721 grouped among a number of tropical New World strains from Brazil, French Guiana, Colombia and Panama in addition to a clade dispersed across Panama, South Africa and Europe. Specifically, UM721 groups with strains CLFT100 from Bahia, Brazil and CLFT111 from Espirito Santo, Brazil (Jenkinson et al., 2016), receiving 99% bootstrap support (Figure 3). Bolivian UM721 grouped separately from the *Bd* strains from Chile (AVS2, AVS4, AVS7) and Argentina (MLA1). In addition, the genome scan for heterozygosity (an inverse proxy for recent, strong selection) showed a very similar pattern of putative recent selection for hypervirulence that we see in other *Bd*-GPL strains (Rosenblum et al., 2013; Figure 4). The genomic heterozygosity profile of UM721 also matches very closely those of its most closely related Brazil isolates (Figure 4).

4 | DISCUSSION

Our results on the genetics of the Bolivian *Bd* strains are both expected and surprising. Because the presence of two *Bd* strains in Bolivia seemed plausible to explain the geographic and temporal pattern of *Bd* occurrence and amphibian declines in the country (Burrowes & De la Riva, 2017a), we expected to find genetic differences between the *Bd* in *T. culeus* from Lake Titicaca and *Bd* in *B. balzani* from the yungas (altiplano vs. Amazonian cloud forests). Surprisingly, this is not the case, and our results suggest that isolates of *Bd* from both *T. culeus* and *B. balzani* belong to the same *Bd*-GPL lineage. Because one of the authors (I. De la Riva) had studied

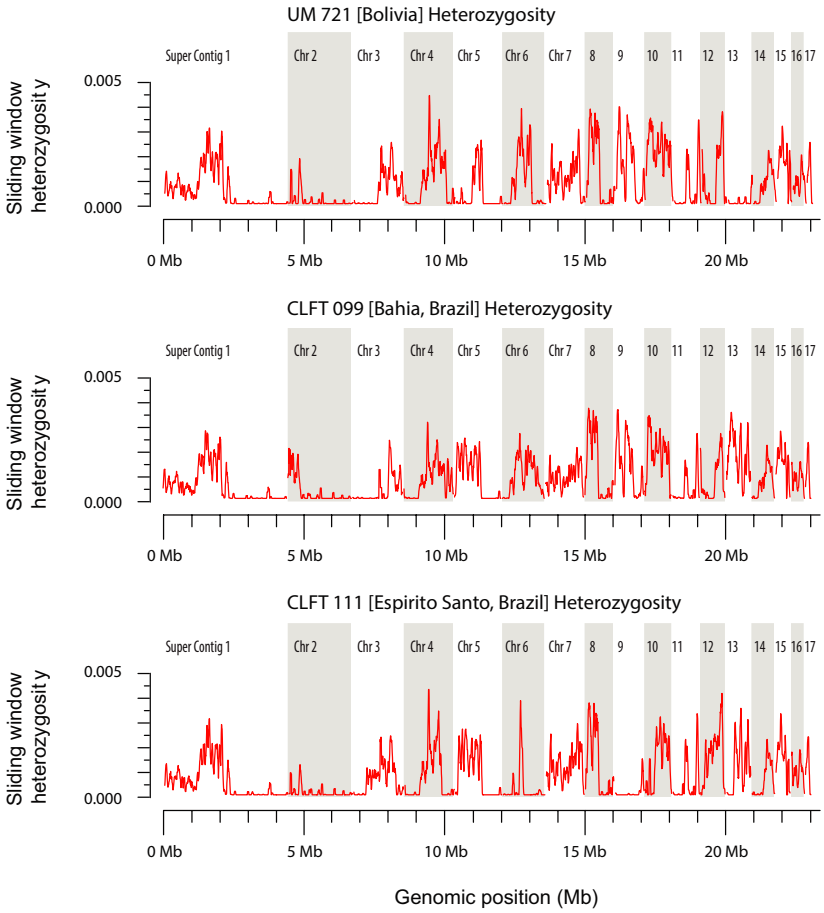

FIGURE 4 Observed genomic heterozygosity based on a 50-kb sliding window analysis advancing every 10 kb for *Batrachochytrium dendrobatidis* strain UM721 isolated from a *Boana balzani* host in the cloud forests of the Bolivian Andes, and its two closest sampled *Bd*-GPL relatives from north-eastern Brazil (CLFT100 and CLFT111)

the rich and diverse amphibian community in the cloud forests of Bolivia during the late 1980s, and witnessed the decline and disappearance of many species starting in the early 1990s (Burrowes & De la Riva, 2017a; De la Riva & Reichle, 2014), we expected to find a hypervirulent *Bd* lineage among persisting frogs in this ecosystem that differed from the genotype of the *Bd* lineage in the altiplano where declines have not been documented.

We also found that the closest relatives to the Bolivian *Bd* strain are Brazilian strains of *Bd*-GPL isolates from extant frogs in the Brazilian Atlantic Forest (Figure 3). Speculatively, we could say that our results indicate that *Bd*-GPL was introduced in Bolivia from Brazil. However, we must be careful in drawing this conclusion because we know that, at present, global sampling of *Bd* isolates (and sampling from South America in particular) is not necessarily sufficient to answer this question. For example, to our knowledge, there are no *Bd* isolates from Peru or Ecuador that might help clarify the relationships of Andean strains and their dispersal pattern.

The presence of the hypervirulent *Bd-GPL* lineage in *Telmatobius culeus* from Lake Titicaca is unforeseen because this species has not declined drastically in Bolivia (De la Riva & Reichle, 2014). In addition, *Bd* was detected in preserved specimens of *T. culeus* collected in this area since 1863 (Burrowes & De la Riva, 2017a), which would put this pathogen in the Bolivian altiplano earlier than in Brazil where it has been detected since 1894 (Rodriguez et al., 2014). Regardless of specific dates, which depend on material available in museum collections, it is evident that in the late 1800s, there were frogs in Brazil and in Bolivia that were already infected with *Bd* and that these species have managed to thrive until present times (Burrowes & De la Riva, 2017a; Rodriguez et al., 2014). However, in the case of *T. culeus*, extant frogs currently struggle not only with lake contamination (Archundia et al., 2017) presumably responsible for mass mortalities in 2009, 2011, 2013 and 2015 (Renick Mayer, 2016), but also with *Bd* infection (Berenguel, Elias, Weaver, & Reading, 2016), which herein we report to be the hypervirulent *Bd-GPL* strain. Thus, we might ask whether these frogs are survivors from a past *Bd* epidemic—what has been coined as 'the ghost of *Bd* past' (James et al., 2015). If this was the case, we would expect *T. culeus* populations to have suffered a massive decline in the past, and extant populations to be a rebound of the surviving individuals. Unfortunately, there are no monitoring records from the past for comparison, but further research might reveal signals of a recent genetic bottleneck (other than the one linked to aridification in the altiplano, which ended around 2600–3800 years ago; see Benavides, 2005). If *Bd-GPL* introduced to Bolivia via the cloud forests in the 1990s has indeed been transported to the altiplano by waterfowl (Burrowes & De la Riva, 2017b; Garmyn et al., 2012; Johnson & Speare, 2005) or other potential vectors, it would explain the recent declines of certain altiplano species such as *Rhinella spinulosa* and *Telmatobius marmoratus* among others (De la Riva & Reichle, 2014), as well as its presence in extant *T. culeus* from Lago Titicaca. This hypervirulent strain (*Bd-GPL*) with its

aggressive infection advantage (Jenkinson et al., 2018) could potentially outcompete older endemic strains and put at risk populations that survived what may have been an old, historically unassessed, *Bd* epidemic ('the ghost of *Bd* past') likely caused by a *Bd* genotype that has been either extirpated, or not detected in this preliminary study.

The presence of *Bd*-GPL in extant amphibian hosts from the altiplano and Amazonian slopes of the Bolivian Andes, as well as the detection of *Bd* in preserved specimens in Bolivia since 1863 (Burrowes & De la Riva, 2017a) and of *Bd-GPL* in Brazil since 1897 (Rodriguez et al., 2014), hint to a much older evolutionary history of this pathogen, and suggests that the origin of the *Bd-GPL* lineage is in the earlier part of the 95% confidence interval (i.e. late nineteenth century; O'Hanlon et al., 2018), or the detection of *Bd* in earlier museum specimens in Bolivia is not *Bd-GPL*, but instead a enzootic genotype that was extirpated or not detected in the present study. There are difficulties in resolving these alternatives because molecular dating is subject to error (Heads, 2005; Tamura et al., 2012), determining the particular strain of *Bd*-positive museum specimens requires complex molecular methods, and there is the added risk that it may be a different chytrid fungus related to *Batrachochytrium*. The invasion of *Bd-GPL* into the cloud forests of Bolivia in the 1990s could have been facilitated by the introduction of trout, movement of aquatic birds (Burrowes & De la Riva, 2017b), or indirectly by the global pet trade which has been linked to the dispersion of pathogenic *Bd* lineages (O'hanlon et al., 2018; Schloegel et al., 2012; Valenzuela-Sánchez et al., 2018). Direct invasion of *Bd-GPL* via pet trade is unlikely in Bolivia since there are no established invasive amphibians in the country (De la Riva & Reichle, 2014) nor a tradition of exotic amphibians kept as pets.

While *Bd* has already affected negatively about 16% of the known amphibian species (Scheele et al., 2019), knowledge of its origin and dispersal history can help us take actions against other pathogens, as has been the case with preventing the spread of *B. salamandrivorans* to the Americas (Gray et al., 2015; Richgels, Russell, Adams, White, & Grant, 2016). Aside from present and historical sampling of *Bd* in museum collections, we encourage efforts to genotype local *Bd* strains where the history of amphibian declines is known. Moreover, the major breakthrough needed is to be able to genotype the *Bd* from older museum collections. These kinds of data allow us to test alternative hypotheses on the evolution of wildlife pathogens, the source of introduction and direction of spread and may help us predict and/or ameliorate the consequences of emergent diseases.

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CONFLICT OF INTEREST

The authors confirm that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study, particularly the genetic sequences of the Bolivian *Bd* strain, are openly available in NCBI at https://www.ncbi.nlm.nih.gov/. The current NCBI accession numbers are also available in the Supplementary Materials section Tables S1–S2 (NCBI, 2020).

ORCID

Patricia A. Burrowes https://orcid.org/0000-0002-3085-6681

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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