RAPID COMMUNICATION

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Genomic epidemiology of the emerging pathogen *Batrachochytrium dendrobatidis* from native and invasive amphibian species in Chile

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Summary
Emerging fungal diseases represent a threat to food security, animal and human health worldwide. Amphibian chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has been associated with catastrophic and well-documented amphibian population declines and extinctions. For the first time, *Bd* was cultured from native and non-native wild amphibians in Chile. Phylogenomic analyses revealed that Chilean isolates AVS2, AVS4 and AVS7 group within the global panzootic lineage of *Bd* (*Bd*GPL) in a single highly supported clade that includes a genotype previously isolated from the United Kingdom. Our results extend the known distribution of *Bd*GPL in South America and suggest a single and relatively recent introduction of *Bd*GPL into the country, providing additional support to the role of anthropogenic activity in the global spread of this panzootic lineage.

KEYWORDS
Batrachyla antartandica, *Calyptocephalella gayi*, chytridiomycosis, emerging infectious disease, fungal disease, *Xenopus laevis*

1 | INTRODUCTION

Anthropogenic activity has contributed to the current rise of fungal diseases, representing a threat to food security, animal and human health worldwide (Fisher, Gow, & Gurr, 2016). Perhaps the most striking example of this problem is chytridiomycosis, an emerging disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*; Longcore, Pessier, & Nichols, 1999). *Bd* occurs in all continents with amphibians, and its impact has been described as "the most spectacular loss of vertebrate biodiversity due to disease in recorded history" (Skerratt et al., 2007). Initially, this pathogen was considered to be a rapidly expanding clonal

lineage with a single geographical origin (Morehouse et al., 2003). However, further molecular analyses comprising more comprehensive methods and a wider geographical representation of isolates revealed a much more complex evolutionary history, diversity and population dynamics (Farrer et al., 2011; Rosenblum et al., 2013). A hypervirulent lineage of *Bd*, termed the global panzootic lineage (*Bd*GPL), is widely distributed across the globe and has been associated with catastrophic and well-documented amphibian population declines and extinctions (Farrer et al., 2011; Rosenblum et al., 2013). In contrast, increasing numbers of endemic genotypes have been isolated from amphibians inhabiting Asia, Switzerland, South Africa and Brazil (Bataille et al., 2013; Farrer et al., 2011; Schloegel et al.,

2012). To date, none of these have been convincingly linked to host population declines.

In Latin America, *Bd* is a recognized threat to many amphibian species (Lips et al., 2006; Soto-Azat et al., 2013; Valenzuela-Sánchez et al., 2017). Despite this, there is a knowledge gap on the molecular diversity of *Bd* genotypes occurring in most areas within this region. For instance in Chile, *Bd* is widespread, infecting native and invasive anurans, but no attempt has been conducted so far to isolate the fungus or to conduct comparative molecular analyses (Soto-Azat et al., 2013, 2016; Valenzuela-Sánchez et al., 2017). This limits our capacity to adequately understand the emergence and epidemiology of chytridiomycosis in the region. The aims of this study are to (i) describe the isolation and whole-genome sequencing of three *Bd* isolates collected from the skin of wild amphibians inhabiting distinct geographical areas of Chile and (ii) to compare these genotypes with a global panel of *Bd*.

2 | MATERIAL AND METHODS

This study was carried out in accordance with the Bioethics Committee of the Universidad Andres Bello (No13/2015), the Zoological Society of London's Ethics Committee (NoWLE709) and the Chilean law (Agriculture and Livestock Service permit No351/2015).

In February 2015, we captured 20 tadpoles of the native marbled wood frog (*Batrachyla antartandica*) in Melimoyu, southern Chile (44°6′31″S, 73°07′06″W; Figure 1a). In May 2015, we captured 10 adults of the invasive African clawed frog (*Xenopus laevis*) in Hualañé, central Chile (34°58′33″S, 71°51′09″W; Figure 1a). Amphibians were kept for no longer than 1 month, with individuals of each species maintained in each of two separated groups. In order to increase the efficiency of the *Bd* isolation process, animals were first confirmed to be infected using a specific real-time PCR assay (Soto-Azat et al., 2013). Infected animals were then euthanized under licence by
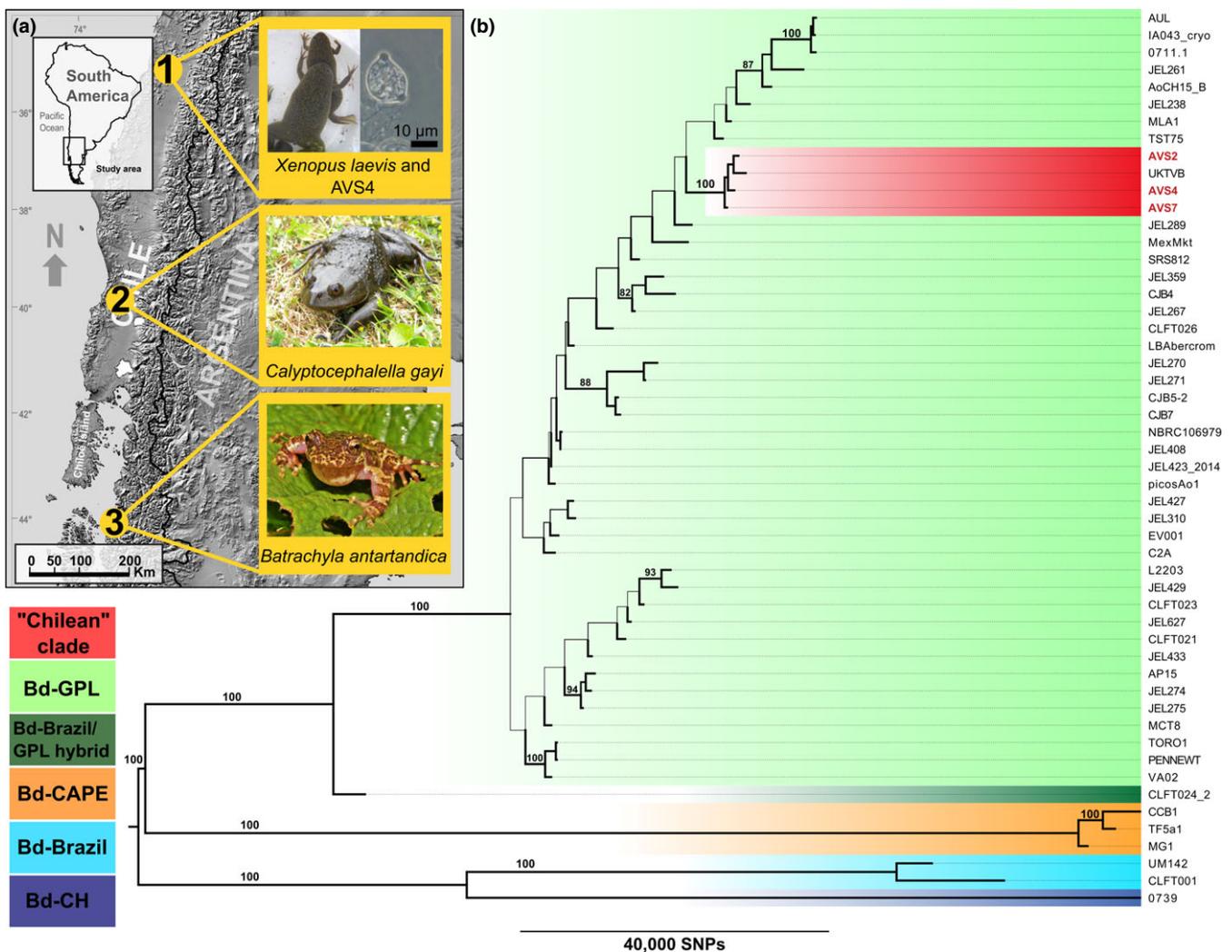

FIGURE 1 (a) Sites of collection, amphibian species captured and *Batrachochytrium dendrobatidis* (*Bd*) [AVS4] isolate obtained from Chile. (b) Global phylogeny of 52 isolates of *Bd* based on 363,497 segregating sites. The clade containing the Chilean *Bd* isolates is highlighted in red, and the Chilean isolates are labelled in red: AVS4 from *Xenopus laevis* from Hualañé, AVS7 from *Calyptocephalella gayi* from Valdivia and AVS2 from *Batrachyla antartandica* from Melimoyu. The branches of the tree are weighted (thickness) by bootstrap support (500 replicates), with branches with 80% of support and above labelled

immersion in a diluted solution of tricaine methanesulphonate (10 g/L) for 1 hr. Once death was confirmed, keratinized tissues of *Bd*-positive animals (i.e., the whole mouthparts of tadpoles and skin sections of ventral hindlimbs and interdigital membranes of adults) were removed and cultured in a fungal growth medium following the protocol developed by Joyce E. Longcore (https://umaine.edu/chytrids/batrachochytrium-dendrobatidis/directions-for-isolation). Additionally, in February 2016, during an extensive survey of *Bd* infection in wild amphibians across Chile, we captured one lethargic larvae of the native Chilean frog (*Calyptocephalella gayi*) in Valdivia (39°48′36″S, 73°15′21″W; Figure 1a). This individual died in the field during the sampling procedure and exhibited partial depigmentation of the mouthpart, a finding consistent with *Bd* infection. Therefore, we immediately transported the carcass (in refrigerated conditions) to the laboratory; culture of *Bd* was performed as described above. Cultures were grown at 21°C in a temperature-controlled chamber and were checked every day for zoosporangia development using an inverted light microscope. We confirmed successful isolation (i.e., presence of free-swimming zoospores and spherical bodies morphologically typical of *Bd* zoosporangia) within 2 weeks. Isolates were passaged no more than three times in order to lessen the chance of genomic change due to prolonged laboratory culture (Voyles et al., 2014) and were subsequently cryo-archived at −80°C following Boyle et al. (2003).

We performed DNA extraction using the MasterPure™ Yeast DNA Purification Kit (Epicentre, Wisconsin, USA). DNA extractions were quantified using a Tapestation 2200 (Agilent Technologies, California, USA) and Qubit 2.0 fluorimeter (Thermo Fisher Scientific, Massachusetts, USA). We prepared DNA samples for sequencing on an Illumina HiSeq 2000 (Illumina, California, USA). TruSeq Nano 350 gel-free sequencing libraries were prepared for 125 + 125 bp paired-end sequencing using the Illumina HiSeq high output V4 chemistry.

Sequencing reads were first cleaned of adapter sequences and quality trimmed using cutadapt v1.10 (Martin, 2011). We mapped the reads to the JEL423 reference genome (GenBank assembly accession: GCA_000149865.1) using Burrows-Wheeler Aligner (BWA) v0.7.8 (Li & Durbin, 2009). We processed the resulting sequence alignment/map (SAM) files using SAMtools v1.3.1 with the "fixmate" and "sort" programs to ready the files for variant discovery. We performed the variant discovery in a two-step process using freebayes version dbb6160 (Garrison & Marth, 2012). In the first step, sorted BAM files for each of the isolates in the phylogeny were independently called to find variant positions. The set of all variant position identified across all samples was merged into a single variant call format (VCF) file. In the second step, each of the samples was re-called using the positions in the merged VCF to produce a squared-off call set (a genotype call is made at every locus for each isolate, including for missing data). All squared-off sample VCF files were processed by vcflib (Garrison, 2016) to break complex variants into allelic primitives and vt (Tan, Abecasis, & Kang, 2015) to normalize short insertion and deletion sequences (indels). We quality filtered the VCFs with bcftools version 1.3.1 (Li et al., 2009) to accept only variants with sufficient supporting evidence: sites covered by 4 reads or less were set to missing; potentially polymorphic sites were additionally filtered

using the settings recommended in the bcbio.variation.recall squaring-off pipeline (Chapman, 2016), with sites not passing these filters set to homozygous reference, that is although there were sequencing reads covering that site, there was not enough evidence to call a variant at that position. We merged the processed VCF files into a single multisample VCF and extracted a fasta file of the SNP variant calls. We conducted the phylogenetic analyses using RAxML v8.2.9 with the GTRCAT model with 500 bootstrap runs. The raw read sequences of AVS2, AVS4 and AVS7 are available at the NCBI Sequence Read Archive: https://www.ncbi.nlm.nih.gov/sra under accession numbers SRR6252317, SRR6252319 and SRR6252318, respectively.

3 | RESULTS AND DISCUSSION

We obtained three isolates across central and southern Chile (Figure 1): AVS2 from *B. antartandica* from Melimoyu, AVS4 from *X. laevis* from Hualañé and AVS7 from *C. gayi* from Valdivia. Phylogenomic analyses showed that all Chilean isolates group within *Bd*GPL in a single, highly supported clade (100% bootstrap support; Figure 1b). The Chilean *Bd* isolates were fixed at 99.4% of the segregating sites in *Bd*GPL after filtering for missing positions. There were only 2,257 variable sites exclusive to the Chilean *Bd* isolates. Although we compared the genomes of AVS2, AVS4 and AVS7 with an extensive global panel of *Bd*, they were shown to be highly divergent from the only known regional endemic lineage (*Bd*Brazil), but clustered with UKTvB, a *Bd* isolate collected from a smooth newt (*Lissotriton vulgaris*) in 2009 in Kent, United Kingdom (bootstrap support 100%, Figure 1b). Inferring phylogenetic relationships within the *Bd*GPL lineage is difficult due to potential SNP homoplasy caused by independent loss of heterozygosity (LOH) events (Rosenblum et al., 2013). We tested the topology produced using SNPs from across the whole autosomal genome against a 1.66 Mb region on supercontig_1.2, which displays a characteristic large-scale loss of heterozygosity (LOH) event, present in all known sequenced isolates of *Bd*GPL (Rosenblum et al., 2013). The topology derived from SNPs in this region (consisting of 19,913 segregating sites) recapitulated the deeply diverged lineages also found in the whole-genome tree, with a very flat comb-like structure in the *Bd*GPL lineage due to a lack of SNPs in this region (Figure 2). All three Chilean isolates in this study formed a clade (albeit with very little bootstrap support) with isolates from Valencia (VA02), Switzerland (AoCH15), the United Kingdom (UKTvB), France (0711.1) and Canada (JEL261), suggesting the clustering with the UK isolate UKTvB in the whole-genome analysis may not be artefact. Finally, we used the Weir and Cockerham's estimator in vcftools to perform a sliding-window comparison of F_{ST} of the Chilean *Bd* isolates against all the other *Bd*GPL isolates. In this analysis, we used a 10Kb window, with a 5 kb step-size along the genome to identify if any stretches of the Chilean *Bd* population were highly differentiated compared to the rest of the *Bd*GPL clade (Figure 3). We identified several stretches of genome where the F_{ST} estimator was more than two standard deviations greater than the
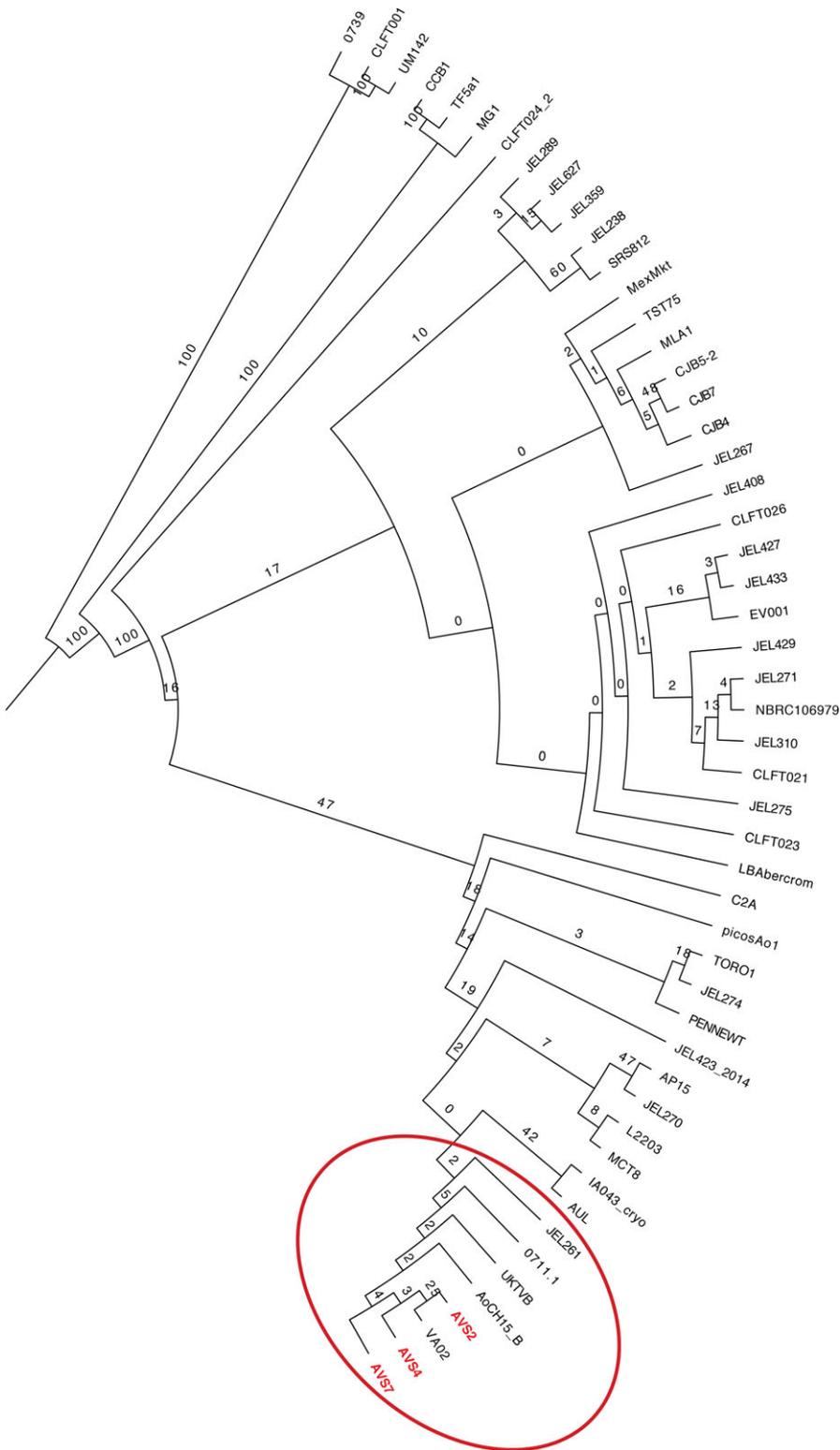
FIGURE 2 A phylogenetic tree of *Bd* isolates with proportional branch lengths, based on a 1.66 Mb region of supercontig_1.2. This region includes a known loss of heterozygosity (LOH) event shared by all know *Bd*GPL isolates. The Chilean isolates (labelled in red) form a clade (circled in red) with several isolates from Europe (0711.1, AoCH15, UKTvB and VA02) and one from Canada (JEL261). The branch labels indicate bootstrap support from 500 bootstrap replicates

mean of all F_{ST} values, indicating differentiation due to positive selection or reduced rates of recombination may be important at these loci in Chile. Further work is required to determine whether any classes of gene are functionally enriched in these high divergence regions.

The low number of segregating sites exclusive to the Chilean *Bd* isolates, compared to the total number of sites where the *Bd*GPL isolates are polymorphic, suggests a single and recent instance of long-distance dispersal of *Bd* into Chile, possibly through the international movement of amphibians, aquatic animals, plants or fomites (Farrer et al., 2011; Schloegel et al., 2012). Molecular characterization of further isolates from Chile and neighbouring countries, along with the calibration of a genome-wide molecular clock, is required to confirm this hypothesis. The existence of a unique, recently
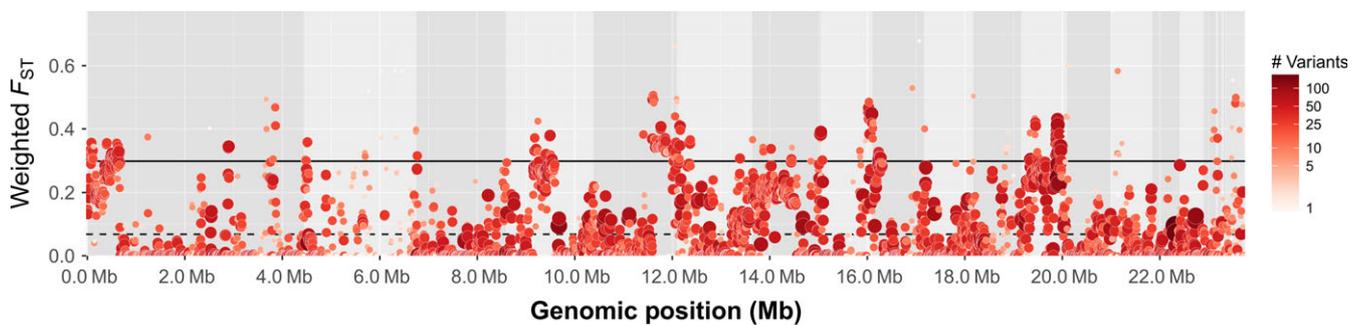
FIGURE 3 Sliding window analysis of population differentiation of Chilean *Bd* isolates against other *Bd*GPL isolates using Weir and Cockerham's F_{ST} estimator. Each point represents a 10Kb genomic window, with a 5 kb step-size. The dashed black line represents the mean F_{ST} (0.0679). The solid black line represents the 95% quantile threshold of the F_{ST} estimator (0.3141). Each point is sized and coloured on a log scale by the number of variants in each window. The legend indicates the colour scale (the number of SNPs included in each window varied from 1 to 177, with a median of 24). Point size from small to large is scaled from low to high numbers of variants

introduced lineage of *Bd* in Chile would be in contrast with the known history of this pathogen in the near Brazil, where both *Bd*GPL and *Bd*Brazil coexist, with evidence of multiple hybridization events between them (Jenkinson et al., 2016). This highlights the importance of biosecurity measures to prevent the introduction and establishment of further *Bd* lineages into Chile, as this pathogen has the capability to increase its genomic diversity through the exchange of haplotypes among lineages.

Xenopus laevis is considered as an effective vector capable of disseminating *Bd* into new regions (Soto-Azat et al., 2016) and thus represents a possible route of introduction of *Bd*GPL into Chile. The earliest evidence of *Bd* in Chile is from a four-eyed frog (*Pleurodema thaul*) individual collected from Concepción in 1970 (Soto-Azat et al., 2013). Although underestimation of *Bd* is a known problem when examining fixed archived specimens (further discussed in Soto-Azat, Clarke, Fisher, Walker, & Cunningham, 2009), hundreds of PCR analyses from archived amphibians have failed to detect *Bd* infections prior to that date in Chile (Soto-Azat et al., 2013). The exact time of introduction of *X. laevis* into Chile is unknown, but it is thought to have occurred sometime in the 1970s (Jaksic, 1998). Additional means of *Bd*GPL introduction, possibly assisted via tourism, research or salmon farming (Liew et al., 2017), should also be considered.

The Chilean *Bd* isolates grouped together in our phylogenomic whole-genome analysis with a genotype isolated in 2009 from the United Kingdom (UKTvB). A similar phylogenetic relationship was observed when restricting the analysis to a subset of the genome spanning an LOH event shared by all *Bd*GPL isolates, with isolates from other European countries and a Canadian isolate also grouping with the Chilean isolates. While there has been no report of mortality caused by the UKTvB, VA02, JEL261 or AoCH15_B isolates, the 0711.1 isolate from the French Pyrenees was associated with amphibian mortalities. In general, *Bd*GPL has been associated with catastrophic mass mortalities and population extirpations in multiple continents (Farrer et al., 2011; Rosenblum et al., 2013; Vredenburg, Knapp, Tunstall, & Briggs, 2010). In Chile, during the last four decades, an extensive extirpation of local populations of the Darwin's

frogs (*Rhinoderma darwinii* and *R. rufum*) has occurred across central and southern Chile. Retrospective, cross-sectional and capture-recapture data suggest that, among other factors, chytridiomycosis has contributed to the occurrence of these extirpation events (Soto-Azat et al., 2013; Valenzuela-Sánchez et al., 2017). The existence of the hypervirulent *Bd*GPL in Chile provides additional support to this hypothesis. The susceptibility of *Rhinoderma* spp. to different *Bd* genotypes, however, requires investigation (e.g., controlled infection experiments), because the host–parasite interaction is a complex process that is affected not only by the infectivity and virulence of the parasite but also by host-specific responses and environmental factors (Woodhams et al., 2011).

This is the first description of Chilean genotypes of *Bd*, and our study gives the first insights into the origin and introduction history of this amphibian pathogen into the country. Our results extend the previously known distribution of *Bd*GPL and provide additional support to the role of amphibian translocations, and perhaps other international movements of aquatic animals, plants or fomites, in the global spread of this panzootic lineage.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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